APPLICATION OF MODERN ANALYTICAL TECHNIQUES AND CHEMOMETRIC METHODS TO THE CHEMICAL CHARACTERISATION OF SOUTH AFRICAN WINES

Determination of Non-Volatiles

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Summary

The present study deals in the first instance with the improvement of current analytical techniques for the analysis of the non-volatile content of wines. An improved sample preparation method, using solid phase extraction (SPE), was initially developed for the analysis of organic acids, sugars and phenolic compounds. Consequently, modern analytical methodologies were assessed to obtain optimal techniques for the separation of various non-volatile compounds. A capillary electrophoresis (CE) method, demonstrably more reliable than currently used high performance liquid chromatography (HPLC) and CE methods, is proposed for the analysis of organic acids. HPLC with refractive index (RI) or evaporative light scattering detection (ELSD) proved more suitable than CE for the analysis of sugars in dry wines. Liquid-chromatography-mass spectroscopy (LC-MS) offered superior sensitivity and resolution compared to the relatively new technique of CE-MS for the analysis of wine phenolics. LC-MS was further applied for the efficient and sensitive analysis of non-coloured phenolics and anthocyanins in wine. Negative- and positive electrospray ionisation, respectively, were used in conjunction with an ion-trap mass analyzer, for the identification of 34 phenolics and 31 anthocyanins in red wine samples. Complementary CE and LC methods were developed to allow the identification of artificial dyes in red wines, added illegally to improve their colour. Also, the application of stir bar sorptive extraction (SBSE) with liquid desorption and micellar electrokinetic chromatography (MEKC) for the analysis of bitter acids in beer is reported.

In the second part of the thesis, the analytical results obtained for South African red and white wines were evaluated. Following comparison of the results with literature reports, several pattern recognition techniques were employed. A classification function obtained by linear discriminant analysis (LDA) was used to classify both red and white wines according to variety, based on their chemical composition. This classification is achieved independent of the factors of wine age or geographical origin, making it useful for authenticity evaluation.

Opsomming

Hierdie studie het as primêre doel die verbetering van bestaande analitiese metodes vir die analise van nie-vlugtige komponente in wyn. In die lig hiervan, is eerstens 'n toepaslike monster-voorbereidingstegniek, gebasseer op soliede fase ekstraksie (SPE), ontwikkel vir die gelyktydige analise van organise sure, suikers en fenoliese komponente vanuit die wyn matriks. Vervolgens is moderne analitiese metodes ondersoek en gepastde skeidingstegnieke is ontwikkel vir die verskillende chemiese wyn-komponente. Kappillêre elektroforese (CE) en hoë-druk vloeistof-chromatografie (HPLC) in kombinasie met verskeie deteksie-metodes is vergelyk. Hieruit is 'n verbeterde CE metode vir die analise van organise sure is ontwikkel, terwyl HPLC in kombinasie met refraksie-indeks- en verdampings lig verstrooiings deteksie (ELSD) die beste resultate lewer vir die analise van suikers in droë wyne. Die toepasbaarheid van vloeistof-chromatografie met massa spektrometriese deteksie (LC-MS) vir die analise van fenoliese komponente is gedemonstreer, terwyl CE-MS onvoldoende resolusie en sensitiwiteit toon vir die analises. LC-MS is vervolgens ook gebruik vir die identifikasie van 34 fenoliese verbindings en 31 antosianiede in rooi wyn. Komplementêre HPLC en CE metodes is ontwikkel vir die identifikasie van onwettige sintetiese kleurstowwe in rooi wyn. 'n Addisionele monster-voorbereidingsstap, roerstaaf sorptiewe ekstraksie (SBSE), is saam met vloeistof-desorpsie en misellêre elektrokinetiese chromatografie (MEKC) gebruik vir die analise van hops bitter sure in bier.

In die tweede deel van die tesis word die resultate verkry m.b.v. bg. tegnieke vir die analise van Suid-Afrikaanse rooi- en wit wyne, bespreek. Die resultate is vergelyk met waardes uit die literatuur, en verskeie statistiese metodes is gebruik om die data te ondersoek. Met behulp van chemometriese metodes is 'n klassifikasie funksie verkry wat die klassifikasie van Suid-Afrikaanse wyne volgens druifsoort, gebasseer op die chemiese samestelling van die wyne, toelaat. Die klassifikasie is moontlik, onafhanklik van die ouderdom of geografiese oorsprong van die wyne.

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Abbreviations

ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionisation
AP-ESI	atmospheric pressure electrospray ionisation
BGE	background electrolyte
CA	cluster analysis
cafta	caffeoyl-tartaric acid
CE	capillary electrophoresis
CEC	capillary electrochromatography
cGC	capillary gas chromatography
CGE	capillary gel electrophoresis
CID	collision-induced dissociation
CIEF	capillary isoelectric focussing
CITP	capillary isotachophoresis
CMC	critical micelle concentration
couta	coumaroyl-tartaric acid
CTAB	cetyltrimethylammonium bromide
CZE	capillary zone electrophoresis
DA	discriminant analysis
DAD	diode array detector
DC	direct current
DTAB	decyltrimethylammonium bromide
EDTA	ethelenediamine-tetracarboxylic acid
EIC	extracted-ion chromatogram
ELSD	evaporative light scattering detector
EOF	electroosmotic flow
ESI	electrospray ionisation
FA	factor analysis
FASI	field amplified sample injection
GC	gas chromatography
HPCE	high performance capillary electrophoresis
HPLC	high-performance liquid chromatography
i.d.	internal diameter
IAD	Indirect absorbance detection
IEC	ion exclusion chromatography
KWV	Ko-operatiewe Wijnbouwers Vereniging van Zuid-Afrika
LC	liquid chromatography
LD	liquid desorption
LDA	linear discriminant analysis
LDL	low-density lipoproteins
LLE	liquid-liquid extraction
LOD	limit of detection
LVSS	large volume sample stacking
MALDI	matrix assisted laser desorption ionisation
MEEKC	microemulsion electrokinetic chromatography
MEKC	micellar electrokinetic chromatography
MS	mass spectroscopy
NAA	1-naphthylacetic acid

NP-LC	normal phase liquid chromatography
PAD	pulsed amperometric detection
PAH	polyaromatic hydrocarbons
PC	principal component
PCA	principal component analysis
PDC	2,6-pyridinedicarboxylic acid
PDMS	polydimethylsiloxane
PPO	polyphenoloxidase
RDA	retro Diels-Alder fission
RF	radio frequency
RI	refractive index
RP-IP-LC	reversed phase ion pair chromatography
RP-LC	reversed-phase liquid chromatography
RSD	relative standard deviation
S/N	signal-to-noise
SBSE	stir bar sorptive extraction
SDB	styrene-divinylbenzene
SDS	sodium dodecyl sulphate
SIM	selected ion monitoring
SPE	solid phase extraction
SPME	solid phase microextraction
TBAB	tetrabutylammonium bromide
TD	thermal desorption
TIC	total ion current
tITP	transient isotachophoresis
TLC	thin layer chromatography
TR	transfer ratio
UV	ultraviolet
UV/Vis	ultraviolet/visible

General Introduction

Aim and Context

The art of winemaking has historically been shrouded in mystery and romance. While recent advances in the scientific knowledge of this age-old beverage have done little to alter this perception, the information gained through numerous studies has allowed not only increased control over the diverse processes involved in producing wine, but has also been of benefit in related fields such as food sciences, microbiology, etc. Knowledge gained on the chemical composition of wine and grapes has proved essential in obtaining these results. Specifically for the final product, wine, recent interest in determining those constituents responsible for the particular characteristics of a wine (quality, potential health benefits) has led to a surge in scientific publications dealing with this aspect. Thus, for example, evidence of the healthful benefits associated with drinking wine in moderation has spurred the elucidation of some of the key compounds present in wine and their physiological affect on the consumer. Another principal aim of the chemical investigation of wine content is the differentiation of wines according to their geographical origin. The motivation, in this case, is to establish "authenticity" of wine samples, in accordance with worldwide protected designations of origin. This chemical information also permits the differentiation of wines according to variety, winemaking practice, and ideally, quality, since this last aspect is generally held to be related to winemaking practice within a specific designation of origin.

Throughout this field of research, the importance of chemistry in general, and analytical chemistry in particular, is evident. Although routine monitoring of wine composition is often performed using non-specific methods, thus allowing only general conclusions to be drawn, a recent trend is the application of state-of-the-art analytical techniques to wine analysis and this has proved particularly fruitful. In the light of this discussion, the aim of the current study was the investigation, using appropriate modern analytical instrumentation, of the non-volatile chemical composition of South African red and white wines. In a related study, undertaken at the same time, similar objectives were set for the volatile composition of the same wines (PhD dissertation of Andreas Tredoux).

The majority of the work was dedicated to the development of robust analytical methods suitable for the determination of a broad range of non-volatile compounds. Considering the nature of the compounds analysed (phenolics, organic acids, sugars), the methods of high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) were used, together with mass spectrometric (MS) and ultraviolet (UV) detection. Choice of each analytical method is based on a thorough review of current applications in the field. Shortcomings of each method are highlighted and improved, where possible. Following the establishment of suitable analytical methodologies, the selected non-volatile constituents were quantified in various red and white wines. Comparison is made between these values obtained for South African wines and those reported in the literature for wines from various other countries. Multivariate statistical methods (chemometrics) were used to analyse the data, and to investigate the possibility of distinguishing between wines from different grape varieties, independent of vintage or origin, based on their chemical content.

The lay-out of the thesis is based on the publications and therefore some repetition could not be avoided. For clarity reasons this was not rectified. Chapters 5, 7 and 12 have been published; Chapters 4 (Chromatographia) and 13 (Electrophoresis) have been accepted; Chapters 8 (European Food Research and Technology) and 9 (Journal of Chromatography A) have been submitted. The manuscript concerning Chapter 11 is in preparation.

1

Introduction

1.1 Historical Introduction

Winemaking has a rich history dating back as far as 6000 B.C., with origins traced back to ancient Near East, presumably southern Caucasia [1]. It is there that the fortuitous proximity of the domesticated wine grape (*Vitis vinifera*) and wine yeast (*Saccharomyces cerevisiae*) combined with the spread of agriculture to allow the production of wine [2]. The natural grape acidity combined with the production of ethanol during fermentation means that wine is relatively resistant to microbial spoilage. This fact, together with the intoxicative properties, probably attributed to the popularity of the beverage. Grape growing and winemaking spread from the Near East to Egypt and Mesopotamia, before reaching Greece, where mention of ancient Greek wines is found in the works of Herodotus and Homer. Greek and Roman poets and historians held these wines in great esteem and presumably the first form of effective maturation, allowed by the use of non-porous amphorae, is responsible for their prestige [3]. Dionysus, the Wine God of Greek mythology, is credited with the invention of wine making, and his association with the product of the vine is the chief attribute of the Roman version of the same god, Bacchus. It is thought that the wines of the Romans were too sweet by modern standards, and flavouring by addition of for example herbs was commonly practised. Winemaking spread through colonisation from the eastern Mediterranean to central and southern Europe, the Roman Empire playing a crucial role in the propagation of this knowledge. Barrels were developed for storage and shipment, while bottles were used for the first time during this period. After the fall of the Roman Empire (500 A. D.), the Catholic Church was largely responsible for the preservation of winemaking technology, although the Arabs contributed scientific irrigation to North Africa and Spain, where the conquerors continued to make wine [4]. However, as preservers of knowledge in the West, the monasteries played a vital role during the Dark Ages. In medieval times wine was consumed daily, since reliable water sources were scarce. This situation was finally alleviated and during the seventeenth and eighteenth centuries the wine industry temporarily declined as a result of competition caused by the availability of distilled spirits, tea and coffee. The use of sulphur in barrel treatment together with the development of better glass-making methods and widespread

use of cork as bottle closures allowed for the first production of "modern" wine during this period. When Louis Pasteur discovered the importance of yeast and bacteria in the 1860's, winemaking practice took on its present form. Even before this time, European exploration led to the global proliferation of winemaking knowledge. In 1769 viticulture was introduced into California from Mexico by Franciscan missionaries, while the first vines were planted in South Africa and Australia in the 17th and late 18th centuries, respectively. Together with South American producers these countries produce the so-called "New World" wines that are only now competing favourably with their European counterparts, previously regarded as far superior.

With the words "Today, praise be to God, wine was pressed for the first time from Cape grapes"*, Jan van Riebeeck heralded the beginning of viticulture in South Africa on 2 February 1659 [5]. These wines were made from cuttings imported from western France. The young wine industry benefited greatly from the arrival, in 1688, of 200 French Huguenots, who settled in outside the Cape peninsula in the regions of Stellenbosch and Franschoek (then referred to as Drakenstein). The sweet wines of Constantia, the estate founded by Simon van der Stel in 1685, became famous in the late 18th century. These wines were in great demand, especially by European aristocracy, for a period of almost 200 years. The affluence of this period is in stark contrast to the situation in the early twentieth century, when epidemics of powdery mildew and *Phylloxera vastatrix* conspired together with global and political forces and severe over-production to cripple the local wine industry. The founding of the Ko-operatiewe Wijnbouwers Vereniging van Zuid-Afrika (KWV), a co-operative of all wine producers, provided much needed stability at this time. Technological and scientific advances made since then have been a major boost to the industry. Thus, Professor A.I. Perold successfully crossed Pinot Noir and Cinsaut (Hermitage) in 1925 to produce the uniquely South African cultivar Pinotage, while the introduction of cold fermentation in 1957 greatly increased the popularity of especially white wines. Consumption of red and white table wines has been increasing since the

* Translated from the original Dutch.

1960's, and the quality of these wines has also been improving, allowing admirable comparisons both New- and Old World wines.

1.2 Economic Importance

Grapes represent the world's most important fresh fruit crop. Grape production is limited to those regions characterised by an approximate Mediterranean-type climate, with major wine-producing countries confined mainly to Western Europe and the Near East, Southern Australia and Africa, and certain countries in North and South America. Worldwide, about 7.4 million hectares were planted under grapes, production of which comprised 61 million metric tons, from which some 26.8 million metric tons of wine was produced, in 2002 [6] (table 1.1). Although South African vineyards account only for roughly 1.5% of the world's total, the country produces 3% of the world's wine (8^{th} in terms of volume produced) [7]. The ratio of white to red grapes planted is roughly 55:45, with progressively more attention being focused on the following five noble cultivars: Cabernet Sauvignon, Merlot, Pinotage, Chardonnay and Sauvignon Blanc [7, 8]. Apart from the revenue created by the sale of wine, some 349 000 people are employed directly or indirectly in the South African wine industry.

Country	Grape Production*	Wine Production*
Argentina	2460	1215
Australia	1754	1220
France	6794	5200
Germany	1425	1018
Italy	7872	4460
Portugal	900	627
South Africa	1350	761
United States of America	6479	2540
World	61018	26787

* Values in 1000's of metric tons, from ref. [5]

Table 1.1: Summary of worldwide grape- and wine production during 2002.

1.3 Scientific Interest

The considerable, and growing, scientific interest in wine is motivated by a number of goals. Primarily, the aim is to improve winemaking technology through broadening of knowledge, with the concomitant financial advantage resulting from competition on the global market. Thus the study of wine begins with the vine, where the fields of climatology, soil chemistry, biology and agriculture are essential to understand the origins of wine characteristics. Knowledge of the physiology and genetics of not only the vine, but also the microbes involved in fermentation and spoilage, is extensively used to improve wine production/quality. In addition, studies on human sensory psycho-physiology form an integral part of oenology. Moreover, a recent surge in scientific interest in wine has been the result of evidence that moderate consumption of wine has health benefits. The inter-disciplinary nature of most research in wine should be noted, with findings being of benefit to other fields of science such as genetics, physiology, etc.

Since the character of a wine at any stage of development is dependent on its chemical composition, this knowledge is crucial to the entire field of wine science. It is thus no surprise that worldwide an extensive body of work has been focussed on elucidating the chemical content of wine and grapes. The knowledge gained in this way since the 1960's has presented the oenologist with a clearer picture of the distinct character of wines, while simultaneously allowing more efficient production of consistent, better quality wines. In this on-going process, chromatographic methods have fulfilled an essential role since the beginning. The development of reliable, quantitative and sensitive analytical methods, in particular gas chromatography (GC) and liquid chromatography (LC), has enabled the rapid identification of new wine compounds. Even though the chemical compounds and the reactions involved in a number of influential processes have been elucidated as a result, much work remains to be done, and the development of suitable analytical methods will continue to play a central role.

1.4 References

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6 FAOSTAT Database (http://apps.fao.org), (2003) Food and Agriculture Organization of the United Nations, Rome

7 WOSA Worldwide Statistics (http://www.wosa.co.za) (2003) Wines of South Africa, Stellenbosch

8 Du Plessis C (Ed.) (2001) SA Wine Industry Directory 2001, Ampersand Press, Cape Town

2

The Chemical Composition of Wine

2.1 Introduction

Wine can roughly be defined as the fermented juice of grapes. This natural beverage consists mainly of water and ethanol. Together with a few additional major compounds, like the organic acids, sugars (fructose and glucose) and glycerol, they are largely responsible for the basic wine taste and mouth-feel [1]. More subtle differences between varietals and vintages can be attributed to the roughly 1 $g \cdot L^{-1}$ of aromatic compounds present. These include higher alcohols and fatty acid esters, although it is largely the phenols, lactones, carbonyls, terpenes, acetals, sulphur and nitrogen compounds, present in minimal amounts, that are responsible for the diverse wine characteristics. During the following more detailed discussion, attention will be limited to the non-volatile wine constituents, as this work is concerned with this fraction.

2.1.1 Water and Ethanol

Water is the predominant chemical component of wine and as such determines the characteristics of this beverage. Thus, compounds have to be at least partially soluble in water to contribute to wine chemistry. On the other hand, the solvent properties of ethanol allow the extraction of tannins, pigments and volatiles, while simultaneously influencing the metabolic activity of microbes and affecting the volatile composition through its chemical reactivity. The significant physiological and psychological effect of ethanol has long been known. Ethanol directly contributes to the sweetness of wines and at the same time modifies the perception of acidity, bitterness and astringency [2]. Moreover, the presence of ethanol is essential for wine stability, and allows its prolonged ageing. Finally, the chemical reactivity of ethanol plays a determinative role in different aspects of oenology.

2.1.2 Sugars

The profusion of fermentable sugars found in ripe grapes is probably responsible for the fact that wine was discovered as the first fruit-based alcoholic beverage. The two principal sugars present in grapes are glucose and fructose (figure 2.1). Grape sugars are produced by photosynthesis, and their concentration increases significantly following veraison – the period when the berries change colour and start to soften. Grapes grown in warmer climates generally reach higher sugar levels as a result of more efficient photosynthesis. The glucose/fructose ratio is used to monitor grape ripening and decreases from about 1.5 at veraison to below 1 at full maturity, i.e. when the total sugar concentration reaches about 20% (w/w). The wine yeast (*Saccharomyces cerevisiae*) derives its metabolic energy from glucose and fructose as the primary fermentable sugars during fermentation. The glucose/fructose ratio decreases further during fermentation as a result of preferential fermentation of glucose. Typically 16-18 $g \cdot L^{-1}$ of sugar is needed for the production of 1 % (v/v) of ethanol [3]. Besides ethanol, compounds contributing to the aroma and flavour such as higher alcohols, fatty acid esters and aldehydes are also produced during fermentation. The sweetness of wine is determined by the residual (unfermented) sugars, consisting mainly of pentoses such as arabinose, rhamnose, xylose and small amounts of unfermented glucose and fructose, the total concentration normally not exceeding 1.5 $g \cdot L^{-1}$ in dry wines. These levels are too low to be detected as sweetness on the palate. The perceptible sweetness of a wine is significantly influenced by other wine constituent such as ethanol and acids, as well as the sugar composition, as fructose is slightly more than twice as sweet as glucose. The concentration of sugars does increase slightly during ageing in oak vats as glycosides present in the wood are broken down [4]. Various glycosides are found in wine, resulting from reactions involving carbohydrates and diverse aglycone molecules. Glycosylated terpenols are varietal aroma precursors. Amongst the various phenolic glycosides, anthocyanins (anthocyanidin-glycosides) are important as they are the compounds responsible for the colour of red wine. For these compounds, glycosylation leads to an increase in stability. Grape polysaccharides are found in small amounts in wine [5], and, apart from their effect on the stability of wine, their contribution to the organoleptic properties are thought to be minimal.

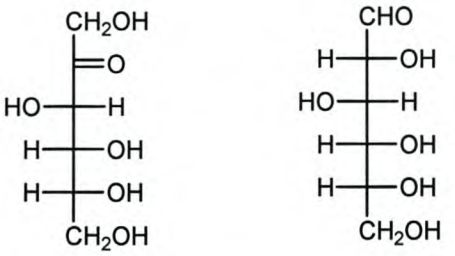

Figure 2.1: Structures of the 2 principal grape sugars, fructose and glucose

2.1.3 Acids

Acids are important structural components of wines. They determine the taste and mouth-feel of a wine, mainly by balancing the sweetness of the alcohol and residual sugars. In red wines the phenolic compounds fulfil a similar role, and thus white wines often require a higher acid content to remain 'balanced'. Acidic compounds are, together with ethanol, largely responsible for the microbial as well as physicochemical stability of table wines. The low pH of wines ensures minimal microbial growth, while simultaneously ensuring colour stability of the anthocyanins, as well as protection against oxidation for phenolic compounds [1]. Acids may also play a role in determining the aroma characteristics of a wine by hydrolysis of acid-labile non-volatile glycosides [6].

Organic acids accumulate in the grape during early ripening, but their concentrations decrease during veraison as the berries swell. The decline in acid levels are dependant on the climate where the grape is grown, as warmer weather leads to more combustion of acids. Thus wines produced in warmer climates are generally characterised by high sugar, and low acid content.

The predominant acids present in grapes are tartaric and malic acid. The occurrence of high concentrations of tartaric acid is a characteristic feature of grapes (in German it is called *Weinsaure*, 'wine acid'), so much so that the presence of calcium tartrate in ancient jars found in the Near East is taken as evidence of winemaking [7]. As a relatively strong acid, tartaric acid is largely responsible for the pH of wine (~3.0 - 3.5). As wine ages, crystallization leads to the precipitation of mainly the calcium tartrate salts. A number of

methods are used to prevent this precipitation from taking place in the bottle, including various cold stabilization methods and addition of crystallization inhibitors [8]. Very few microorganisms metabolize this acid and as a result this compound is essential to the stability of a wine.

Malic acid, the main acid in apples, is found in large amounts in unripe grapes. The concentration rapidly decreases during maturation of the grape following veraison though, both as a result of respiration of the cells, and as result of the increase in the berry volume. Excess loss of malic acid in warmer climates can lead to wines with a flat taste and low stability, while in cooler regions wines with a sour taste (too much malic acid) are sometimes produced.

Citric acid is the third most prevalent acid found in grapes, although at concentrations (0.5 - 1 $g \cdot L^{-1}$) far below those of tartaric and malic acids. Apart from the organic acids, grapes also contain minimal amounts of phenolic acids (hydroxycinnamic acid and benzoic acid derivatives), which will be discussed at a later stage.

A number of acids are also produced during fermentation. Succinic acid is a by-product of yeast metabolism, entering the wine during fermentation. Acetic acid is mainly responsible for the so-called volatile acidity of wines, and is produced in small amounts during fermentation. At these levels the presence of acetic acid is considered beneficial, while excess acetic acid is indicative of bacterial spoilage. Lactic acid is only formed in small amounts during fermentation. However, lactic acid bacteria are capable of decarboxylating malic acid, leading to the substantial amounts of lactic acid, in a process referred to as malolactic fermentation. The net effect is the replacement of the harsher malic acid by the softer (and less acidic) lactic acid. Malolactic fermentation is commonly used as a tool to reduce the acidity of especially red wines from cooler climates, where higher acid amounts could lead to tart, sour wine. The structures of the six principal acids found in wines are presented in figure 2.2.

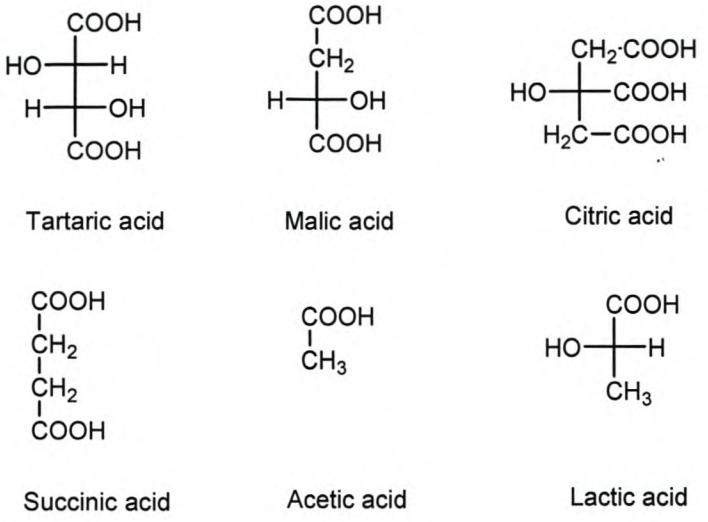

Figure 2.2: Structures of the six principal organic acids found in wine.

Additional organic acids present in wine include isocitric, fumaric, pyruvic, citramalic and oxaloacetic acids, most of which are minor constituents not known to contribute to sensory properties.

2.1.4 Phenolic compounds

The phenolic composition plays a determinative role in the quality of especially red wines, and to a lesser extent white wines. These compounds essentially affect the taste, colour, mouth-feel, fragrance, antioxidant and antimicrobial properties of wine. Phenols originate from the grape and vine stems as well as from wood cooperage and are produced by yeast metabolism. Phenolic extraction from the grape is dependant on many factors, such as the temperature and duration of fermentation, and the phenol content of wine shows greater variation than almost any other wine constituent. In addition, significant changes in the phenolic content of wine take place during ageing.

2.1.4.1 Non-Flavonoids

The phenolic content of wine can principally be divided into two classes: flavonoids and non-flavonoids. The non-flavonoid group consists mainly of phenolic acids, i.e. the benzoic acid and cinnamic acid derivatives and the stilbene derivatives. The benzoic acids found in wine differ from each other with regard to the number and pattern of substitution of hydroxyl- and methoxy groups on the aromatic ring. Examples are 3,4,5-trihydroxybenzoic acid (gallic acid, figure 2.3) and 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid). These compounds are released from their respective glycosides, the predominant form found in grapes, by acid hydrolysis.

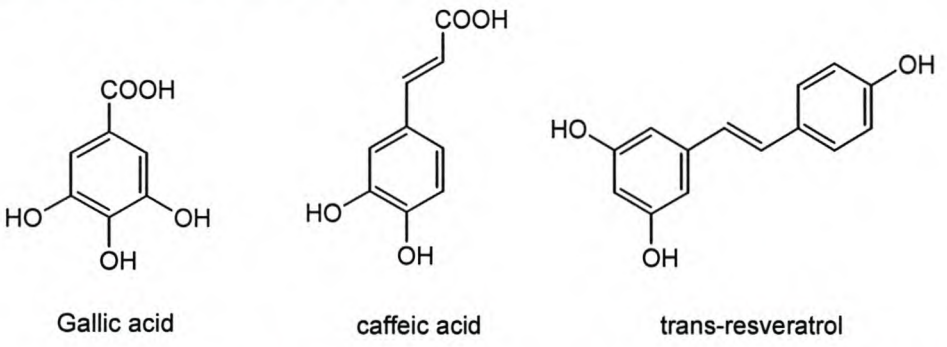

Figure 2.3: Examples of non-flavonoid phenolics present in wine: a benzoic acid derivative (gallic acid), a hydroxycinnamic acid (caffeic acid) and a stilbene (trans-resveratrol).

The hydroxycinnamic acids are important constituents of wine because of the role they play in enzymatically initiated oxidation reactions. These compounds are found in the juice and the pulp of the grapes and, as such, are the main phenolic constituents of white wines prepared without pomace contact. Substitution of the aromatic ring by hydroxyl- and methoxy groups also provides variety within the group (figure 2.3). Cinnamic acids are often found esterified with especially tartaric acid (for example coumaroyl tartaric acid), producing compounds very susceptible to oxidation and causing oxidative browning of white wines [9]. Caffeic acid and p-coumaric acids are also found coupled to anthocyanin glucosides in red wines. The cinnamic acid derivatives are important from a metabolic point of view, since p-coumaric acid is a precursor in the synthesis of both the flavonoids and another class of phenolic compounds in wine, the stilbenes [10]. Phenolic acids do not

directly contribute to wine taste, with the exception of gallic acid and its polymeric form, tannic acid, which have been shown to be bitter and astringent [11]. Also, the phenolic acids are known precursors of volatile phenols produced by micoorganisms [12, 13].

Stilbenes have been the focus of a lot of scientific research recently. Amongst the stilbenes found in wine, resveratrol (trans-3,5,4'-stihydroxystilbene, figure 2.3) is a known phytoalexin, produced in the vine in response to fungal infection. Resveratrol is synthesised particularly in the skin cells, from where it is extracted into wine during fermentation, resulting in higher concentrations in red compared to white wines [14]. Interest in this compound and its presence in wine is the result of evidence of beneficial physiological properties [15, 16]. Since the detection of this compounds in wine [17], the presence of other forms of trihydroxystilbenes have also been reported [18-20]. These include cis-resveratrol as well as the glucosides of both isomers (the polydatins).

2.1.4.2 The Flavonoids

The flavonoid structure is based on a C6-C3-C6 skeleton, and this class contains several groups, which differ in the oxidation level of the central heterocyclic ring (figure 2.4). Wine flavonoids can be divided into three groups in order of increasing oxidation state of ring C: flavanols, anthocyanidins and flavonols. Within these groups, diversity arises from substitution patterns at rings B and C by hydroxyl, methoxy, prenyl-, geranyl- and sugar residues, as well as their ability to exist in oligomeric or polymeric form [21].

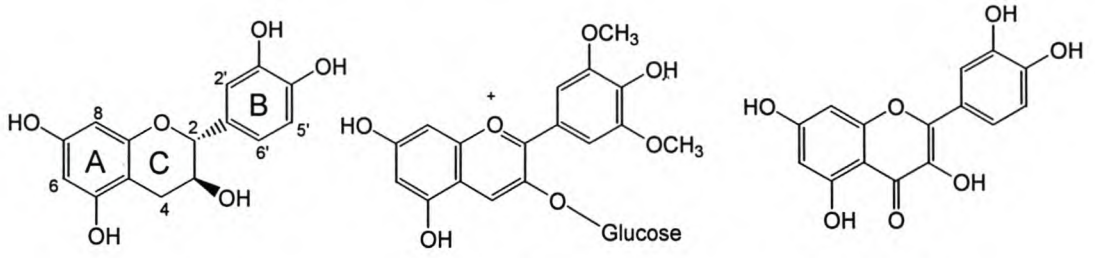

Figure 2.4: Representative compounds of the three classes of flavonoids found in wine: flavanols, anthocyanins and flavonols.

2.1.4.2.1 Flavanols

The flavanols are colourless compounds that are found in monomeric, oligomeric and polymeric form in wine. The primary monomeric flavanols found in grapes and wine are (+)-catechin (figure 4) and (-)-epicatechin, and, in contrast to flavanols and anthocyanins, they do not occur in glycosylated form. Both compounds have been shown to be bitter as well as astringent [22]. Grape tannins are generated by the polymerization of flavanol precursors, and can be divided into procyanidins, consisting of (epi)catechin units (1,2-dihydroxyphenyl ring B), and prodelphinidins, incorporating (epi)gallocatechin units (1,2,3-trihydroxyphenyl ring B). Monomeric units are linked through C4-C8 and/or C6-C8 bonds (the so-called B-type procyanidins), and may contain 3-O-esters of gallic acid (galloylated procyanidins). Grape seed tannins are partly galloylated procyanidins [23, 24] while skin tannins also contain prodelphinidins [25]. The average molecular weight of the skin tannins is higher than those of the seed, whereas seeds contain more tannins and larger proportions of galloylated units. Apart from the proanthocyanidins mentioned above, wine can also contain hydrolysable or pyrogallic tannins, consisting of polymers containing ellagic acid and/or gallic acid with glucose [1, 26]. These compounds do not originate from the grape, but are extracted from oak cooperage.

Tannins are defined by their capability to cross-link proteins, and there is evidence that a certain polymer size is required for the interaction to be effective. This phenomenon leads to coagulation of mucin in saliva by rendering the glyco-proteins therein insoluble. This effect of tannins, together with their ability to stop secretion by salivary glands and to harden mucous tissue, is responsible for the sensory perception known as astringency. Astringency is thus perceived as a dry, puckering sensation throughout the oral cavity, to be distinguished from the taste sensation bitterness. Bitterness, also caused by tannins and other phenolic compounds, is registered by the circumvallate papillae located at the back of the tongue, only after swallowing in the case of drinking and as an aftertaste in the case of tasting [27]. The organoleptic properties of tannins are dependant on their structures, and skin tannins are regarded as being 'softer' than seed tannins. The lower molecular weight flavanols, as well as gallic acid, are both bitter and astringent [2, 11], while procyanidins of increasing molecular weight become more astringent and less bitter. Moreover, bitterness and astringency are influenced by molecular conformation (epicatechin is more bitter and

astringent than catechins), and are affected by other wine components and/or properties, including viscosity, pH and ethanol percentage [2]. During wine ageing, the more bitter monomers and dimers are polymerized, leading to an increase in the astringency and a concomitant decrease in the bitterness of the wine. Ultimately, further polymerization will lead to insoluble polymers, and their precipitation will lead to a decrease in astringency.

During winemaking, catechins and oligomeric proanthocyanidins are extracted from grape seeds and stems (if present), while polymeric proanthocyanidins are contributed by the stems and skins [28]. Mixed procyanidin-prodelphinidin as well as pure gallocatechin oligomers have recently been detected in wine [29]. Following extraction from the grape, polyphenols are gradually degraded. However, the total amount of phenols in wine stays constant, indicating that they are converted to other species [21]. In contrast to the production of tannins in grapes, which is under enzymatic control, reactions involving tannins in wine are less controlled. Consequently these reactions generate modified tannins, degrade some existing ones and generate new ones. Several reactions involving tannins take place in wines: acid-catalysed bond making and bond-breaking [30], as well as oxidation reactions [31, 32], leading to eventual precipitation of insoluble polymers. Also, acetaldehyde-induced polymerisation of flavanols has been reported in model solutions and wine, involving flavanols [33] and/or anthocyanins [34, 35] as co-reactants. Glyoxylic acid, formed by oxidation of tartaric acid, can replace acetaldehyde in this reaction, leading to the formation of non-coloured polymers linked through carboxymethane bridges, as well as several yellow xanthylium salts [36-38]. Further reactions involving anthocyanins and/or flavanols will be discussed at a later stage.

The enzyme polyphenoloxidase (PPO) catalyses the oxidation of mainly o-diphenolic substrates to o-quinones in the first step of the process responsible for enzymatic browning. The most important PPO substrates in wine musts are caffeoyltartaric acid and to a lesser extent p-coumaroyltartaric acid. Both are converted to caffeoyltartaric acid o-quinone, which can undergo oxidation by powerful reductants such as ascorbic acid or sulphite ions, or coupled oxidation of other o-diphenols, regenerating cafeoyltartaric acid [9], or condensation reactions involving hydroquinones (figure 2.5) [39]. In addition, however, primary and secondary o-quinones can undergo addition reactions involving nucleophilic phenolic compounds [21]. When the product of these reactions has a lower redox potential

than the original hydroquinone, its further oxidation is favoured, and in this way some of the original phenol remains unoxidised. This regenerative polymerisation is encouraged by slow oxidation, and responsible for the formation of diverse polymeric phenolics during wine ageing [39]. These enzymatically-catalysed oxidation reactions are prevalent during the early stages of winemaking, but as the amount of oxygen and enzymatic activity decrease, the addition reactions involving flavanols and anthocyanins become predominant.

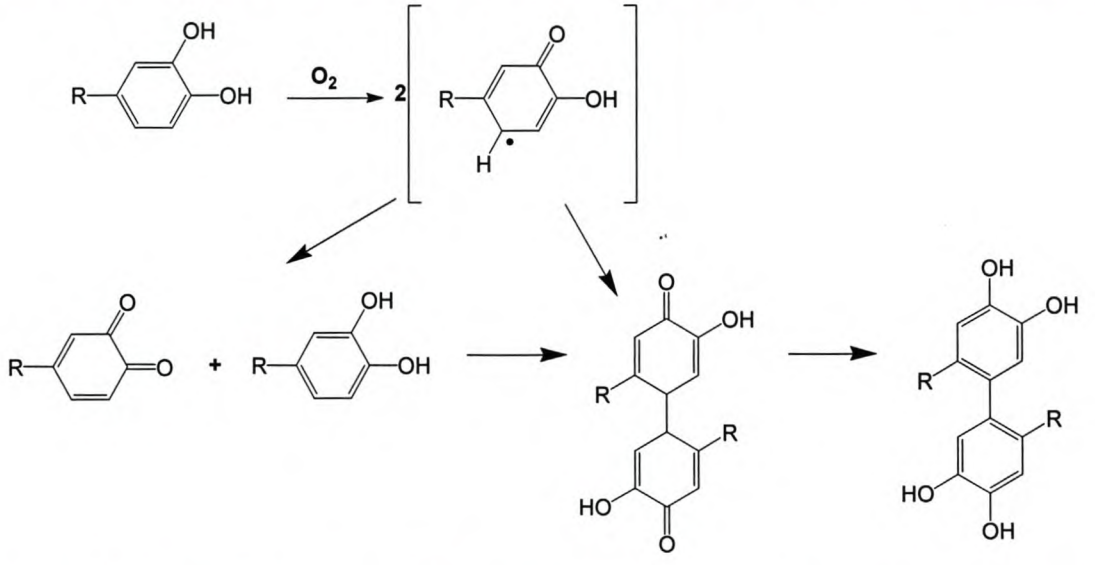

Figure 2.5: A simplified example of the regeneration of an oxidizable oligomeric substrate by reaction between two semi-quinones or a quinone and a phenol [39].

The capability of tannins to form stable combinations with proteins and polysaccharides, mainly through hydrophobic interaction and hydrogen bonds, often leads to precipitation of these complexes due to lack of solubility [1]. This phenomenon is used to remove undesirable wine components, including phenolic compounds, during the fining process [40]. Furthermore, interactions between phenolics and aroma substances may also affect the flavour of wine [41].

2.1.4.2.2 Anthocyanins

Anthocyanidins, when attached to a sugar molecule, form the more stable anthocyanins (figure 2.4), the components responsible for the colour of red wine. The aromatic character of ring C allows sideways overlap of the orbitals of all three rings and the resulting absorption of green light at approximately 520 nm gives wine the characteristic red colour. Further, acylation of the sugar group with hydroxycinnamic acids such as p-coumaric acid may occur (except in Pinot Noir), and this is thought to stabilize the red flavylium cationic form of anthocyanins. This cationic form occurs in pH-dependant equilibrium with a number of other chemical species. Loss of a proton from the cationic species may occur at a phenolic hydroxyl group, generating a quinoidal base. This quinoidal base is blue, its formation being favored by high pH. A second, more important, reaction involving cationic anthocyanins is the addition of water to ring C (the attack occurs at positions 2 or 4) and the subsequent loss of a proton, generating the colourless hydrated form (figure 2.6). At wine pH less than half of the total anthocyanin pigment is in the coloured flavylium form because of this equilibrium. Here production of the colourless hydrated form is once again favored by an increase in pH [42]. A third reaction detrimental to the red colour of wine is the addition of bisulfite (this is the principle form of free sulfur dioxide at wine pH) by exclusive attack at position 4 of ring C, generating a colourless hydrogen sulfite addition product. This reaction is most important since the sulfite ion is much more reactive than water towards the positively charged flavylium cation [43]. The extent of formation of the hydrogen sulfite addition product depends upon the concentration of free sulfur dioxide, which is often added to wine to prevent oxidation and microbial growth [44].

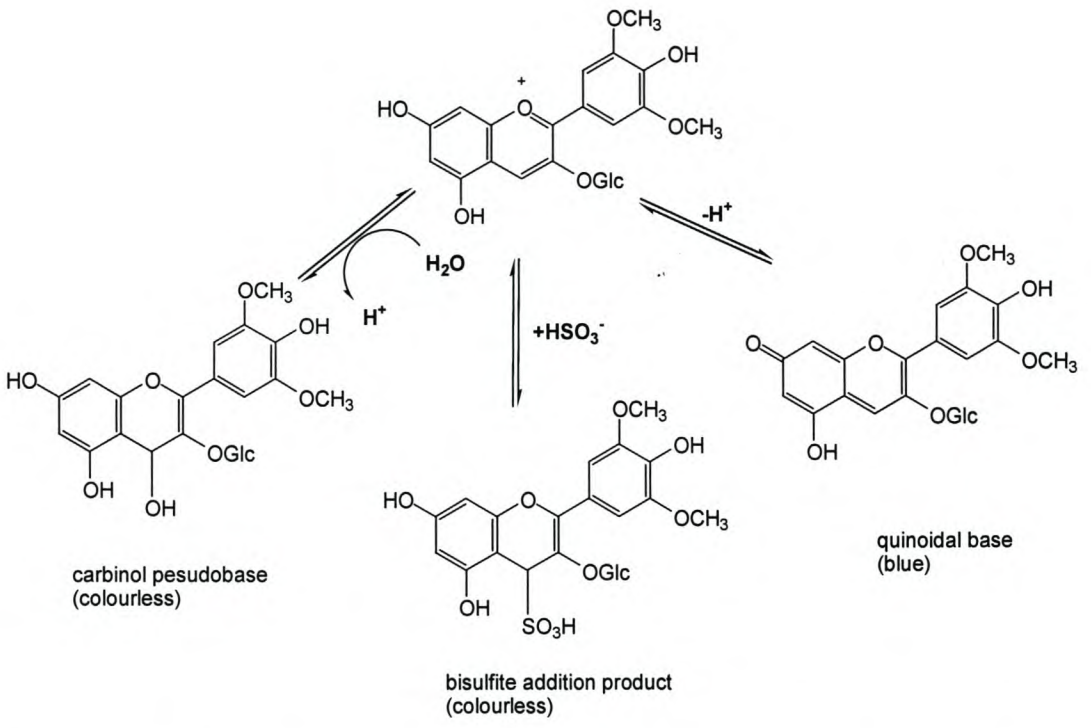

Figure 2.6: Equilibria between the various forms of malvidin-3-glucoside found in wine.

Anthocyanins are extracted from the skin of black grapes during maceration to colour the young red wine purple-red. Free anthocyanin concentration in wine, initially as high as 1500 $mg \cdot L^{-1}$, rapidly decrease to trace amounts found in old wines as a result of condensation as well as degradation reactions [42, 45]. This process is responsible for the change in colour (from purple-red to brick-red) as well as the loss of astringency observed during wine ageing [30]. Intense research focussed on the anthocyanin content of wine has revealed a variety of mechanisms by which the free anthocyanins are converted to more stable pigments over time.

Degradation reactions normally involve either thermal degradation, where the anthocyanin equilibria are irreversibly shifted towards non-coloured forms, or oxidative degradation with light and oxygen acting as catalysts. Furthermore, non-covalent interaction between anthocyanins and other phenolics, known as co-pigmentation, influences the colour of the young red wine, and might be the first step in the formation of pigmented condensed tannins [46, 47].

Somers [42] suggested the direct condensation of anthocyanins with tannins as a reaction responsible for the changes in pigment content of ageing wine. Since then, evidence of these reactions has been obtained in model solutions [30], and more recently the reaction products have been identified in wine [48, 49]. Either molecule can act as electrophile, allowing two possible reaction mechanisms. A second reaction takes place in acidic medium, following the reaction of an ethanal carbocation with flavanol/procyanidin or anthocyanin nucleophilic sites (the latter in neutral carbinol base form). The reaction product in this case contains a flavanol/procyanidin unit linked via an ethyl-bridge to an anthocyanin (figure 2.7). The formation of these compounds was suggested by Timberlake and Bridle [34], and demonstrated in model solutions [33] and wine [49-51]. The flavanol molecule involved in this reaction could also be oligomeric in nature, and products resulting from acetaldehyde mediated addition of procyanidin B2 to malvidin-3-glucoside have been reported [35]. Because of the numerous wine compounds capable of taking part in these reactions, a broad spectrum of products is expected in wine medium.

Cycloaddition of various wine constituents possessing polarisable double bonds to the anthocyanin C4 have been reported. These include the covalent binding of 4-vinylphenol [52-54], as well as a number of related compounds [55], to acetylated as well as non-acetylated anthocyanins. Recently, it has been shown that the reaction mechanism involves interaction between intact hydroxycinnamic acids and anthocyanins [56]. The reaction of volatile compounds such as 4-vinylphenol with anthocyanins is likely to alter the flavour of wine in addition to modifying the phenolic composition. Addition of pyruvic acid to acetylated and non-acetylated malvidin produces the compounds named vitisin A by Bakker et *al.*, as well as a number of related compounds [57-59]. In a similar reaction, anthocyanins undergo condensation with acetaldehyde, producing vitisin B and similar products [60]. It would seem that the 5-OH present in grape anthocyanins is a prerequisite for the chemical changes taking place during wine ageing, a phenomenon unique to wine amongst as beverage [61].

All of the mentioned reaction products are more resistant to increase in pH and bisulphite bleaching [35, 52, 60]. Additionally, these compounds are coloured orange-red due to shifts in λ_{max}-values to lower wavelengths. In a young wine, the contribution of these derived pigments to wine colour is overshadowed by the presence of grape anthocyanins, but during

ageing the free anthocyanins are lost due to degradation and condensation reactions, resulting in the tile-like red colour observed in old wines. In fact, the contribution of derived pigments to wine colour is as high as 50 % after 1 year in the bottle, and increases up to an average value of 80 % after 10 years [42].

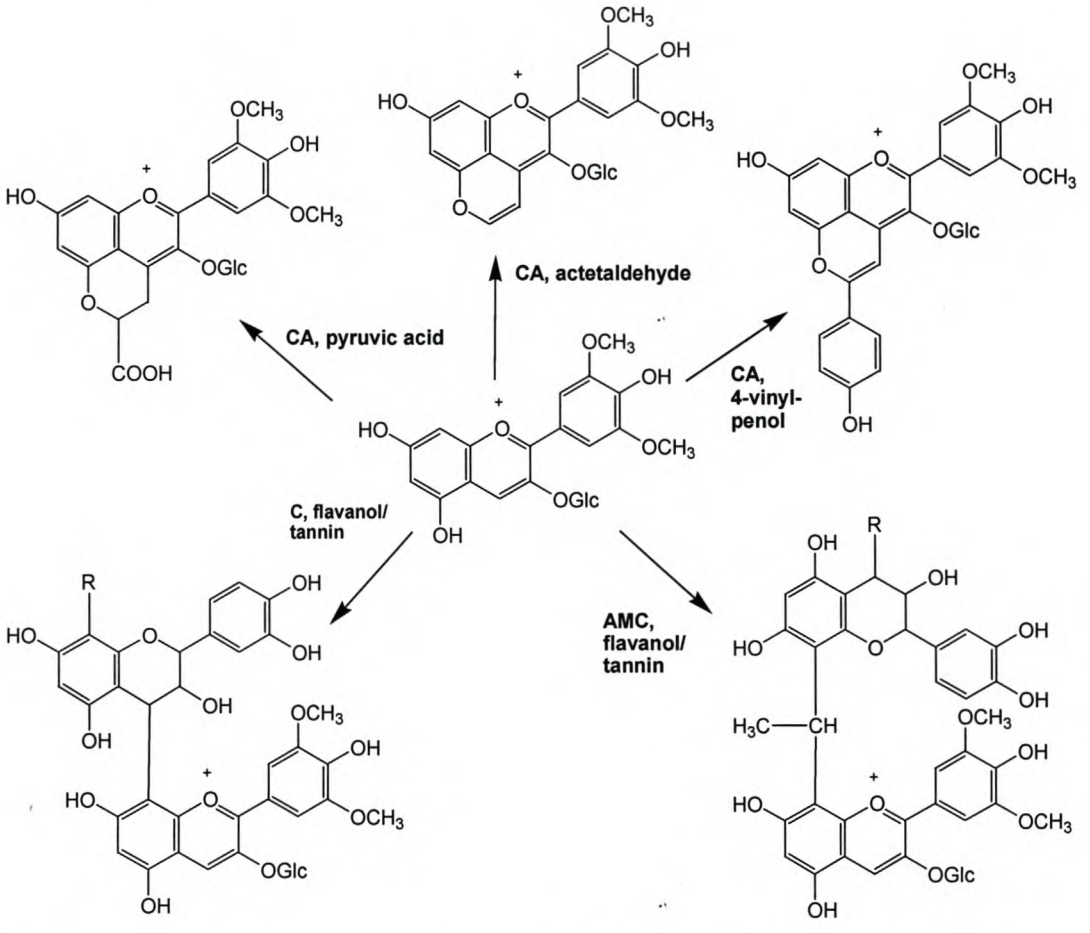

Figure 2.7: Chemical reactions involving malvidin-3-glucoside taking place in wine. R = H or part of a tannin structure, CA = cyclo-addition reaction, C = condensation reaction, AMC = acetaldehyde mediated condensation reaction.

Not only do the changes in the anthocyanin composition affect the colour of a red wine, the organoleptic profile is altered as a result of concomitant changes in wine tannins, in a manner that is only vaguely understood. In support of this fact, significant correlations

between wine quality ratings and colour densities have been reported for young Australian and French wines of the same variety and style [62, 63]. Interestingly, the quality ratings were not correlated with the total anthocyanin concentrations, but with their degree of ionisation. In a study involving French Cabernet Franc wines, it was noted that "grapes yielding intense and balanced wines were characterised by high anthocyanin to tannin ratios" [64]. The tannin quality was thus dependant on, amongst others, the tannin polymers and tannin-anthocyanin polymers, their ratio being determined by the nature and amounts of precursors. It is evident that the exceedingly complex nature of the phenolic content of especially red wine plays a determinative role in the quality of the wine. As a result of this complexity, however, relation of the phenolic content to sensory properties is problematic. Further investigation into the nature of the reactions involving these compounds, their relative contribution to phenolic composition, as well as the sensory characteristics of the various products is essential to obtain a better understanding of wine-making and of wine itself.

2.1.4.2.3 Flavonols

The third class of flavonoids, the flavonols, is widespread in the skins of both red and white grapes. Mainly three flavanols are found in red wine grapes: kaempferol, quercetin and myricetin, while only the first 2 are present in white grapes. These compounds occur in glycosylated form within the grape, with the aglycones being released into the wine by hydrolysis occurring during fermentation [1]. Minimal amounts are found in white wines as a result of limited skin contact during fermentation. Flavonols are light yellow compounds believed to protect plant tissues from damage by ultraviolet light. Quercetin (figure 2.4) may also contribute to the colour of white wines.

Finally, mention should be made about the relatively recent surge in research into phenolic compounds in general, and the phenolic content of wine in particular, that is the result of evidence of the beneficial effect of certain phenolics on human health. The so-called "French Paradox", the dietary anomaly responsible for the low risk of heart disease of the French, despite their high consumption of saturated fats [65], has largely been responsible for the interest in the health benefits of wine.

The nutritional value and antimicrobial activity of wine have long since been known, but the more recent interest is focussed on the evidence of reduced risk of cardiovascular disease related to wine antioxidant properties, as well as anti-tumour activity of wine constituents. Although the protective effect associated with drinking wine in moderation can largely be attributed directly to the favourable effect of ethanol on circulating lipids and on avoiding blood clotting, thus reducing the risk of coronary heart disease (CHD), several studies have revealed that especially red wine was more potent than spirits or beer at reducing CHD. The phenolic content of wine is widely held responsible for this feature associated with red wine since it is at least an order of magnitude lower in white wines and virtually absent in other alcoholic beverages [66, 67].

The flavonoids have been shown to be potent antioxidants due to their ability to act as free radical scavengers and in this way to protect cells against oxidative stress [68]. Anthocyanins, flavanols, and wine phenolics as a whole have been shown to inhibit oxidation of low-density lipoproteins (LDL) [69-72] and also to prevent the cytotoxic effects of oxidised LDL [73]. Resveratrol has attracted particular attention because of its potent antioxidant and antifungal activity, and further investigation showed that this compound has many potentially beneficial biological uses [16]. Other potent antioxidants include quercetin and tannin subunits such as catechin. Inhibition of platelet aggregation by phenolics has been reported, and may contribute to lessen the risk of a heart attack or stroke [1]. In addition, the moderate consumption of wine can reduce the risks of certain cancers. This can at least partially be attributed to antiproliferative effects of certain flavonoids [1, 74].

2.1.5 Minerals

Aside from the non-volatile organic compounds discussed above, wine also contains inorganic material in the form of inorganic anions and cations. The mineral content mostly originates from vine accumulation, which is determined by various factors such as soil composition and climatic influences. However, the use of fertilizers and pesticides increases the levels of phosphates, nitrates, as well as some cations such as copper,

manganese and zinc [75]. Also, the acidity of wine is responsible for dissolving metals such as copper, nickel, zinc and lead by corrosion of winery equipment.

The most prevalent cation present in wine is potassium, while less calcium, sodium, magnesium, iron and copper are present. Levels of copper and iron are especially important, as excess of either of these metals can lead to precipitation following reactions involving proteins present in wines (referred to as copper and iron *casse*, respectively). In addition, copper may catalyse oxidation reactions [1], while iron, in the presence of oxygen, is involved in the oxidation of phenolic compounds [76, 77]. Although the metal content of wine is important from a stability and safety point of view, and has been investigated as indicative of the geographical origin [78], metals do not contribute directly to the organoleptic properties of wine.

2.1.6 Nitrogen Compounds

2.1.6.1 Amino acids

Aside from minimal amounts of mineral nitrogen present in musts (fermenting grape juice), amino acids are the predominant form of nitrogen found in grape juice and wines, and as such are essential yeast nutrients during fermentation. Following fermentation, proline is the prevalent amino acid [79], as a result of the inability of yeast to assimilate this compound. The increase in free amino acid content during ripening can largely be attributed to an increase in the proline and arginine concentrations. Levels of these compounds are used as markers for grape ripeness [80, 81], while the ratio of arginine to proline is determined by the grape variety [82]. In fact, amino acid profiles have been used to differentiate wines according to variety, origin and vintage [83]. Apart from their role during fermentation, amino acids are precursors of a variety of aroma compounds found in wine, formed through microbial metabolism of amino acids [84-86]. Here the role of cysteine as precursor for sulphur-containing volatiles should be noted [87]. The amino acid content of musts and wine has also been related to the production of the carcinogen ethyl carbamate [88]. It is unlikely that amino acids directly affect the taste of wine because of their relatively low concentrations [1].

2.1.6.2 Bio-amines

These non-volatile amines, including the well-studied histamine, are known to have physiologically detrimental effects if present in sufficient amounts. Bio-amines are primarily formed by decarboxylation of amino acids. Lactic acid bacteria are largely responsible for the production of bio-amines in wine [89], leading to higher values in red wines, in the order of less than 5 ppm, compared to white wines [90]. Higher levels, indicative of bacterial spoilage, may lead to values in excess of the maximum allowable 10 $mg \cdot L^{-1}$ [80].

2.1.6.3 Proteins

In addition to amino acids and small amounts of peptides [91], wines also contain proteins, originating from the grape. These compounds are responsible for the instability of especially white wines, where their flocculation or precipitation (so-called protein *casse*) affects the clarity of the wine. Red wines contain minimal amounts as a result of precipitation of free proteins with tannins. Bentonites (aluminium silicate clays) are commonly used to eliminate unstable proteins, together with other unwanted compounds such as bio-amines, from wine [80].

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3.1 Introduction

Chromatography was pioneered more than a century ago by the Russian botanist Mikhial Tswett, when he successfully separated plant pigments using calcium carbonate as stationary phase [1]. Following this discovery, Martin and Synge introduced liquid-liquid partitioning chromatography in 1941 [2], and James and Martin published the first paper on gas-liquid chromatography in 1952 [3]. In the ensuing decades theoretical as well as practical developments have led to the establishment of gas chromatography (GC) and liquid chromatography (LC) as the pre-eminent analytical methods worldwide. In this chapter an overview of the chromatographic techniques relevant to this work is presented.

3.2 High Performance Liquid Chromatography

Liquid chromatography (LC) refers to that form of chromatography where analytes are separated by differential partitioning between a moving liquid phase and a stationary phase. The name high performance liquid chromatography (HPLC) is used to distinguish the sophisticated instrumental form of liquid chromatography where high pressure is used to push a sample (and mobile phase) through a column packed with small particles (2-10 μm), from classical gravity flow liquid chromatography and thin layer chromatography (TLC). HPLC is currently the most widely used analytical separation technique because of its suitability for the analysis of non-volatile and thermally labile molecules, which comprise *ca.* 80% of known molecules.

3.2.1 Efficiency in HPLC

In HPLC the separation efficiencies are inherently lower compared to GC due to the slower diffusion in liquids in comparison with gases [4]. However, compared to GC, HPLC offers much more possibilities to tune the column selectivity and an extensive amount of

stationary and mobile phase combinations have been described in the last decades. Nevertheless, efficiency can be optimised in HPLC and an overview of the parameters affecting it is described here.

Zone broadening in HPLC is the result of the following phenomena:

- Eddy diffusion, which is a result of the multiple pathways a solute can follow through a packed bed,
- Longitudinal diffusion in the mobile- and stationary phases.
- Resistance to mass transfer, a consequence of the time required for solute molecules to diffuse from the interior of one phase to the surface where transfer can occur.

Chromatographic band broadening within the column can generally be described by the van Deemter equation:

$$H = A + \frac{B}{u_o} + Cu_0 \qquad (1)$$

$$\sim 2\lambda d_p + \frac{2D_M}{u_0} + \frac{f(k)d_p^2}{D}u_0 \qquad (2)$$

where H is the theoretical plate height (zone dispersion per unit length), u_0 the mobile phase velocity, d_p the particle diameter, λ the packing factor, f(k) a function of the capacity factor k and D the diffusion coefficient representing diffusion in both the mobile and stationary phases [5]. Additional extra-column band broadening occurs in the dead volume outside the column itself. This volume includes the volume of tubing connecting the column to the injector and detector as well as in the injector and detector devices themselves [6].

The effect of the mobile phase flow rate on efficiency can be seen in H *vs.* u curve (figure 3.1). From this plot it is evident that $H_{min} \approx 2d_p$ at the optimum flow velocity (a result that can be derived mathematically for the case of silica particles, the most common packing used in LC). The number of theoretical plates (N) can thus be related to the particle diameter, d_p, and the length of the column (L) according to:

$$N = \frac{L}{2d_p} \qquad (3)$$

It seems obvious that the efficiency of an HPLC column can be dramatically increased by increasing the length of the column, or by decreasing the particle size of the packing material. However, the pressure drop limits the overall column length and thus efficiency, resulting in plate numbers for liquid chromatography being an order of magnitude smaller than those achieved in gas chromatography. Using specialised equipment in a method termed ultra-high pressure liquid chromatography, the group of Jorgenson have demonstrated this principle, achieving in excess of 200 000 theoretical plates on a capillary packed with 1 μm particles [7]. In practice, when using commercial instrumentation, the maximum practical column length for 5 μm particles is 25 to 30 cm.

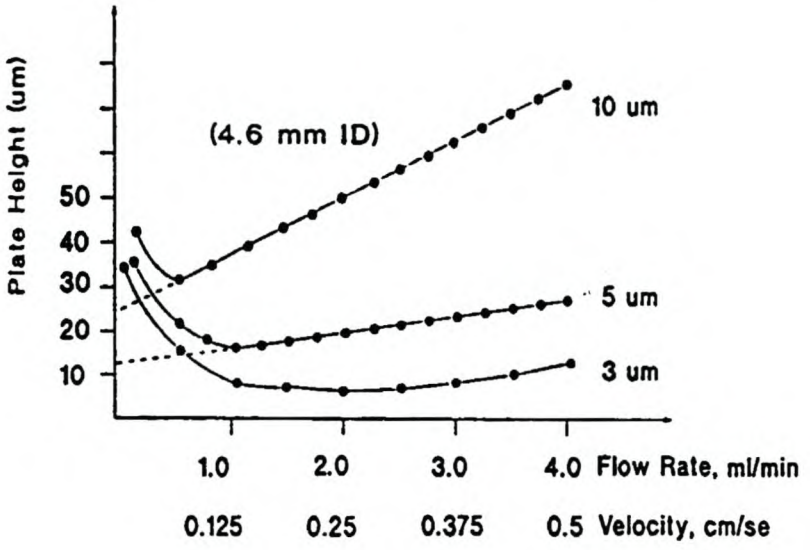

Figure 3.1: Plot of theoretical plate height (H) versus mobile phase velocity (u) for HPLC columns packed with different size particles.

From experimental and theoretical H-u curves constructed for different particle sizes (figure 3.1), it is also evident that the increase in slope (i.e. the efficiency decrease) at higher flow rates is a function of the particle size [8,9]. Thus, higher efficiencies are possible at higher flow rates using particles smaller than 3 μm, thereby opening the possibility of fast HPLC applications. Furthermore, reduction in column diameter can be used advantageously to

decrease solvent consumption with concomitant increase in sensitivity (less dilution of analytes occurs). Once again, however, a compromise has to be made between efficiency, speed, sensitivity and the pressure drop over the column.

3.2.2 Selectivity in HPLC

From the discussion above it is clear that modern HPLC suffers from relatively poor efficiency compared to GC. As mentioned, however, HPLC offers increased selectivity and both the stationary and mobile phase can be varied. This aspect has been exploited extensively, allowing separation of complex mixtures using a variety of interactions. As a result, a number of LC methodologies, differing in the type of chemical interactions involved, have been developed. Details of the most common forms of modern HPLC are discussed below.

3.2.3 Modes of HPLC

3.2.3.1 Normal Phase LC

This was the form of liquid chromatography used by Tswett (hence the name "normal"), sometimes also referred to as adsorption chromatography. A polar stationary phase (silica gel is the most common adsorbent) and an apolar mobile phase (organic solvents, such as hexane, isopropanol) characterise normal phase LC (NP-LC). The affinity of the solute for the adsorbent is determined by its polarity, i.e. the nature of functional groups. The main drawback of adsorption chromatography is the adsorption of especially water originating from the mobile phase, resulting in variable surface composition and irreproducible retention times [10]. This also greatly limits the use of gradients for complex samples [11]. A solution to this problem is chemical modification of the silica surface, leading to replacement of the active silanol functions with a chemical functionality of choice. The chromatographic process is now one of partitioning instead of adsorption. In fact, bonded phase partition chromatography is nowadays the most widely used form of liquid

chromatography, surpassing adsorption chromatography, ion exchange chromatography and size exclusion chromatography. In the case of normal phase (partition) chromatography, polar moieties are bonded to the silica particles and apolar mobile phases are used. Modification is based on the use of aminopropyl-, cyanopropyl- and diol functionalities. Primarily the polarity of the solute determines the distribution between the stationary and mobile phases.

3.2.3.2 Reversed Phase LC

Reversed phase HPLC (RP-LC) refers to that form of bonded phase chromatography where apolar groups (primarily octadecyl, octyl and phenyl groups) are attached to the silanol groups on the surface of the silica gel used as support. In reversed phase HPLC the mobile phase is relatively polar (normally aqueous mixtures of organic modifiers such as methanol, acetonitrile, and tetrahydrofuran) and eluent strength is increased at higher organic modifier content. Retention is primarily the result of non-specific hydrophobic interaction between the solute and the stationary phase [10].

The popular reputation of RP-LC is the result of the simplicity, versatility, ruggedness and general applicability of the method. The latter advantage stems from the fact that most organic molecules possess hydrophobic functions capable of interacting with the stationary phase. Polar, non-polar and ionic compounds have been successfully separated. This is made possible by the diverse nature of the mobile phases that are compatible with this form of LC. Finally, the number of applications in the literature and availability of reliable column technology add to the attractiveness of RP-LC, making it the most popular form of LC in use today.

Reversed phase methodologies allow the addition of various additives to the mobile phase in order to tune the selectivity. Particular mention will be made of ion pair LC. This form of liquid chromatography is used to analyse polar, charged analytes on reversed phase columns. This approach entails the addition of an organic counter-ion (normally in the form of a surfactant present below it's critical micellar concentration) to the mobile phase. Through electrostatic interaction of the surfactant with the analyte, the retention is selectively increased. Selectivity can be tuned by choice of the counter ion, allowing better

separation of certain ionic mixtures than can be achieved by ion exchange LC (e.g. mixtures of analytes with similar pK_a values). The ability to separate charged as well as neutral sample components, as well as the use of high efficiency reversed phase columns are additional advantages of this method [12].

3.2.3.3 Ion Exchange LC

This is the liquid chromatographic method routinely used for the separation of ionic, water-soluble analytes. Differential retention is achieved when analytes have dissimilar affinity for stationary phase ionic groups immobilised on a macromolecular matrix (e.g. styrene-divinylbenzene copolymers). The mobile phase normally consists of an aqueous solution of an electrolyte. Mobile phase ions compete with analyte molecules for the active (ionic) sites. Thus, anion exchange is performed when the anionic component of the electrolyte acts as counter-ion for the positively charged stationary phase cationic groups, and is selectively replaced by the negatively charged analyte [13]. The charge and solvation volume of each analyte directly determines its affinity for the stationary phase. Additional non-specific interaction between analytes and the stationary phase matrix (e.g. hydrophobic interaction) also affect the retention [10]. Selectivity of an ion exchange separation can be manipulated in the first instance by choice of a suitable pH: analytes to be retained should be maximally charged (pH gradients are also used to separate complex mixtures). Moreover, the operating temperature has a significant bearing on the selectivity of the separation, as the chemical equilibria involved are temperature dependent.

Ion exclusion LC is a related technique where acidic or basic compounds are separated based on a Donnan exclusion mechanism instead of ion exchange. Similar stationary phase packings are used, but in this case the charge is the same as that of the analyte. In the simplest case, retention is determined by the access each analyte has to the pore volume of the packing material, with highly charged solutes being excluded and thus eluting first. In fact, a number of different interactions play a role in most of these separations, including partitioning, size exclusion and ligand exchange [14, 15].

3.2.4 Instrumental Aspects of HPLC

3.2.4.1 The Liquid Chromatograph

In order to provide reasonable flow rates when working with column packings consisting of 3- to 10 μm particles, high pressures are required, which places certain demands on HPLC equipment. In the first instance, the pumping system is required to generate pressures of up to 40 MPa with a pulse-free output while being able to produce flow rates ranging from 0.1 to 10 mL/min with sufficient reproducibility. There are a variety of pumping systems able to meet these requirements; the most widely used is the reciprocating pump [16]. This pump consists of a pump chamber, inlet- and outlet check valves, and a piston connected to a camshaft. With each stroke of the piston a small volume is displaced from the pump chamber, with the check valves ensuring flow of the mobile phase in the correct direction during intake (filling) and delivery strokes. The pulsed flow produced by this pump can be limited by a number of approaches. In the case of dual-head reciprocating pumps, the pumping profiles of the two pumps overlap, leading to cancellation of pulses. An alternative approach is to work in series, with the second piston acting as a pulse compensator. Most reciprocating pumps also work in series with a pulse dampener, normally in the form of a compressible fluid separated from the mobile phase by a diaphragm. Electronic feedback is used to control motor speed, for further smoothing of pulsations, as well as to ensure constant flow at different column backpressures. Advantages of this pump include small internal volume, high output pressure and adaptability to gradient elution [17-19]. Other pump systems are displacement pumps consisting of syringe-like chambers equipped with a plunger driven by a stepping motor, and pneumatic pumps where the pressure from a gas cylinder delivered through a large piston drives the mobile phase. The latter pump is inconvenient for gradient elution [17, 20].

A gradient programmer is needed to change the composition of the mobile phase (either continuously or stepwise) during gradient elution. Low-pressure gradient programmers (where the gradient is formed ahead of the pump in use), consist of a proportioning valve with a micro-mixing vessel to promote complete mixing of different solvents.

Alternatively, the output from two or more high-pressure pumps is programmed into a low-volume mixing chamber.

Another important requirement of HPLC equipment is the need to introduce the sample onto the pressurized column as a narrow plug, thereby avoiding peak broadening. A widely used injection system is based on sampling loops in a six-way valve, which allows sample injection without significant interruption of the flow [13, 15, 17]. Rotation of the valve either removes the sample loop from the flow path to allow injection of the sample at ambient pressure, or can rapidly place the sample-filled loop in the mobile phase flow path (figure 3.2). Modern instruments routinely contain auto-samplers allowing reproducible injection of almost any volume of sample. Apart from automated sequential injection, these instruments offer possibilities for automated dilution, derivitisation and standard addition [17].

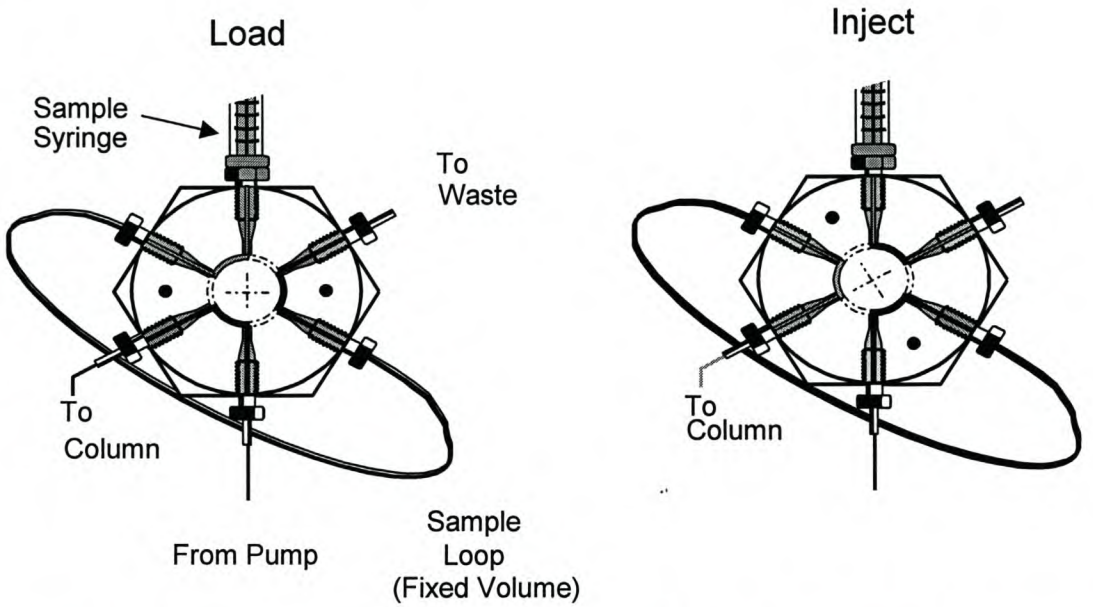

Figure 3.2: Schematic diagram of the sample loading and injection positions of a 6-way valve routinely used for HPLC injection.

Liquid chromatographic columns are constructed from smooth-bore stainless steel tubes to withstand high pressures. Standard analytical columns range in length from 10 to 30 cm with an internal diameter of 4 to 5 mm. Wider bore columns are used for preparative

purposes, while short (3-7 cm) conventional-bore columns packed with 3 μm particles decrease analysis time as well as solvent consumption. Narrow bore columns (1-2 mm i.d.) are increasing in popularity, and offer the advantages of lower solvent consumption, greater sensitivity (less dilution), and compatibility with mass selective detectors. A short guard column is often introduced before the analytical column to increase the life of the analytical column by removing particulate matter as well as sample components that interact irreversibly with the stationary phase [8, 10, 21].

Most commercial instruments are equipped with column heaters to control the temperature of the eluent. This makes it possible to control column temperatures to within a few tenths of a degree, an essential attribute when working with temperature-sensitive detectors such as the refractive index detector.

Finally, mention should be made of the recent trend aimed toward miniaturisation of instrumentation to ensure compatibility with narrow bore columns. Specialised instrumentation, where extra-column band-broadening is kept to a minimum, is needed to reap the benefits of these columns.

3.2.4.2 Detectors

The role of the detector in HPLC is to continuously monitor the column effluent. As such the requirements of an ideal HPLC detector include adequate sensitivity, a stable, reproducible and linear response over a wide range of analyte concentrations, a limited detector volume to limit zone broadening and a fast response time. A distinction can be made between bulk- and solute property detectors (or non-specific and specific detectors). In the former case, the detector response is determined by changes in some physical property of the mobile phase and solute, with reference to the mobile phase only (e.g. the refractive index detector). Solute property detectors, on the other hand, respond to some physical or chemical property of the analyte itself (for example UV detectors), ideally independent of the composition of the mobile phase [17]. At present there is no truly universally sensitive HPLC detector and selection of detection mode is decided based on the separation problem at hand.

3.2.4.2.1 Refractive Index Detector

The refractive index (RI) detector was, along with the fixed wavelength detector, the first detector used in HPLC in the 1970's. The RI detector responds to changes in the refractive index (velocity of light) of the column effluent, and is capable of detecting any solute with a refractive index significantly different from the mobile phase. This operating principle means that the detector is extremely sensitive to small changes in the solvent composition, precluding the use of gradient elution. Detector noise originates from various factors: dissolved gases in the mobile phase, slight changes in mobile phase temperature, or incomplete mixing of the mobile phase. As a result, the primary advantage of the RI detector, namely the universal response, is offset by its poor sensitivity.

Three types of commercially available RI detectors can be distinguished. For a deflection-refractometer the signal is the result of the bending of a light beam as it crosses a dielectric interface at a non-zero angle of incidence. The dielectric interface is found between the sample and reference cells, and changes in the refraction index of one cell will result in the bending of the beam. The Fresnel refractometer measures the reflected light at a glass-liquid interface, detecting in this manner changes in the refractive index of the liquid. The interferometric refractometer measures the interference of two light beams after they were passed through the sample and reference cells, respectively. Changes in the speed of light, caused by differences in refractive index between the two cells, are detected as a result of destructive interference causing a reduction in the energy reaching the photomultiplier. All three instruments have advantages and disadvantages [17, 22]. However, despite improvements in sensitivity and stability of RI detectors since the 1970's, this form of detection is normally the method of choice only when no other detector is suitable.

3.2.4.2.2 Ultraviolet Detector

The absorption of radiation in the ultraviolet and visible region of the electromagnetic spectrum is the result of quantized transitions between electronic levels of the analyte. These transitions are the result of an electron being promoted from an occupied molecular orbital to an unoccupied one of higher energy. For organic molecules absorption occurs

over a wide range of wavelengths because they have many excited modes of vibration and rotation at room temperature. The molecule can thus undergo electronic and vibrational-rotational excitation simultaneously, and since the spectrophotometer is not able to resolve the closely spaced absorption lines, the UV spectrum consists of a broad band centred around major transitions [23]. According to the Beer-Lambert law the absorbance ($=\log(I_0/I)$ where I_0 and I are the intensities of the electromagnetic radiation entering and leaving the sample, respectively), is related to the molar absorptivity (ε), the concentration of the solute (c) and the path length of the sample cell (l):

$$A = \varepsilon c l \qquad \textbf{(4)}$$

The popularity of UV/Visible detection can be ascribed to its simplicity, ease of use, relatively low cost and its versatility [17]. It is the most widely used detector in both liquid chromatography (about 65% of used detectors) and in capillary electrophoresis (CE). The fairly universal application of UV detectors stems from the fact that most non-volatile organic molecules contain polar functional groups absorbing electromagnetic radiation in the UV region. However, sensitivity depends strongly on absorption wavelength, as most compounds absorb maximally in the region 180-210 nm, similar to common HPLC mobile phases.

Based on their ability to provide single or multiple wavelengths, three different UV detectors for HPLC can be distinguished. Fixed single wavelength detectors consist primarily out of an atomic vapour lamp, a simple filter and a photodiode for detection. In the so-called forward optics arrangement, the pre-selected wavelength passes through the detection cell and is read by a single diode. The type of lamp used determines the wavelengths available for detection, the most common being mercury, cadmium or zinc atomic vapour lamps.

The variable wavelength detector also employs the direct optics set-up, but a light source covering a broad band of wavelengths is combined with a monochromator or grating for wavelength selection. Wavelengths can be selected over the range 190-800 nm when making use of deuterium (UV lamp, 190-350 nm) and tungsten (visible lamp, 350-800 nm) lamps. Full UV-Vis spectra can be recorded on-line at a rate of about 10/s by rotation of the

In liquid chromatography, a linear response range of 10^4 to 10^5 and detection limits in the most favourable cases in the range of 0.01 ppm are possible with UV detection. In the design of the detector flow cell a compromise has to be made between minimising extra-column band broadening (by reducing the cell volume), and maximizing the path length to increase sample detectability. For conventional columns cell volumes of 10 μL and a path length of ~10 mm are most often used [13, 16, 17, 22].

Certain structural information can also be gained from UV spectra. As examples, flavonoids have two major absorption maxima, one in the range of 240-285 nm (band II, originating from the A-ring benzoyl system), and one in the range of 300-400 nm (band I, originating from the B-ring cinnamoyl system). Anthocyanidins (and anthocyanins) are notable exceptions with band I absorption maxima in the range 465-550 nm, and band II being represented by a less intense peak in the 270-280 nm region. These absorption bands are due to $\pi \rightarrow \pi^*$ transitions, with the wavelength of maximum absorption varying according to the substitution pattern of the particular flavonoid [24, 25].

3.2.4.2.3 Evaporative Light Scattering Detector

The evaporative light scattering detector (ESLD), as one of the more recent additions to the array of HPLC detectors, has been proposed as a universal detector to replace the RI detector [26-29]. Compared to the latter detection method, ELSD offers improved sensitivity and compatibility with gradient elution [26].

The principle of detection is based on the nebulization of the column effluent to form an aerosol, followed by solvent evaporation, and detection of solute droplets in a light scattering cell (figure 3.4). In the nebulizer a stream of nebulizing gas (normally nitrogen) is used to create an aerosol. The volatile constituents of this aerosol are then evaporated in a heated drift tube prior to entering the light scattering cell. The latter consists out of a laser light source, a light trap (directly opposite the laser) and a photodiode at a 90° angle from the laser. The light scattered by the solute particles is detected by the photodiode to provide the ELSD signal [30]. The only requirement for a solute to be detected by ELSD is that it should be less volatile than the mobile phase employed. The amount of light scattered, and thus the detector response, is determined by the solute particle size, which is in turn

determined by the solute concentration, nebulizing gas flow rate, and mobile phase flow rate. Particle sizes increase at higher mobile phase flow rates, but this can lead to incomplete evaporation of the mobile phase and an increase in noise. Thus, for a specific flow rate, there is an optimal drift tube temperature and nebulzing gas flow rate that produces the most favourable signal-to-noise ratios.

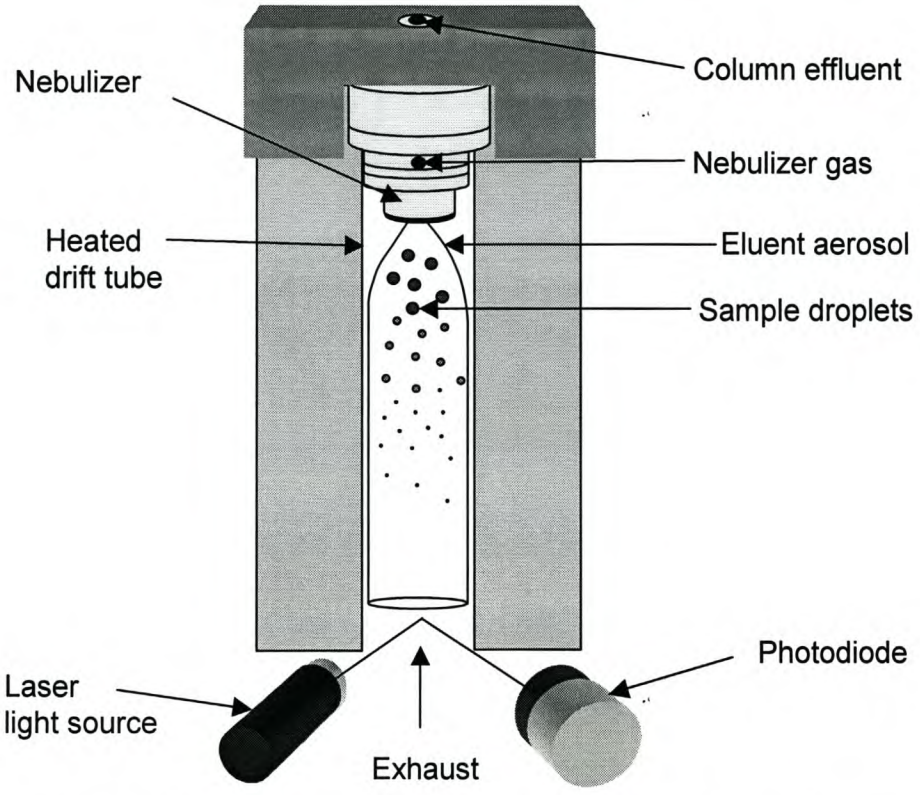

Figure 3.4: The basic components of an evaporative light scattering detector.

The greatest drawback to the use of the ELSD has been the lack of linear response of the detector [8,31]. Although the signal formation has not been properly explained, a combination of nebulization and light scattering theories predict that the ELSD response cannot be truly linear over a significant concentration range [29].

3.2.4.2.4 Mass Spectroscopy

The coupling of mass spectroscopy (MS) to any separation technique provides the best combination of sensitivity and structural information presently available. The mass spectrometric process involves three steps: creation of gas phase ions, separation of these ions according to their mass-to-charge (m/z) ratios, and finally quantification of ions at each m/z value. A mass spectrum therefore allows the analyst to obtain information on the molecular weight, while simultaneously providing structural information of the compound analysed. Since J. J. Thompson performed the first MS experiments in 1912, experimental and theoretical advances have made MS one of the most powerful tools available to the analyst. The coupling of MS to GC has long been an important analytical tool, and with the more recent developments allowing the hyphenation of MS to liquid-based separation methods, the utility of MS has been greatly increased. In the following discussion, the focus will be on description of LC-MS theoretical and practical basics.

The ionisation of organic molecules for MS can be achieved in a variety of ways. Similarly, a number of interface methods have been used for the, often problematic, coupling of LC to MS, each designed with a specific ionisation method in mind. The more important techniques, used in this study, are atmospheric pressure chemical ionisation (APCI), and atmospheric pressure electrospray ionisation (AP-ESI), and both modes will be discussed briefly.

In the AP-ESI process, the first step is the nebulization of the column effluent, which occurs at the tip of a nebulizing needle, with concentric high-pressure gas around the needle assisting the process (figure 3.5). The fact that nebulization occurs without heating of the eluent is at least partially responsible for the 'soft' ionisation observed in ESI-MS. A large potential difference is applied between this nebulizing needle and a surrounding semi-circular electrode. The electric field in the ionisation chamber causes ions of one polarity to be attracted to the surface of the droplets, resulting in the formation of a fine spray of charged droplets, called electrospray [32]. A counter flow of heated nitrogen gas (drying gas) from the transfer capillary serves to reduce the size of the droplets and to remove all non-charged material. The charged droplets continue to shrink until the point where the repulsive electrostatic forces within the droplet become stronger than the surface tension of the droplet, and the droplet explodes in a process referred to as Coulombic explosion. The

droplets continue to shrink by consecutive explosions until sufficient surface-charge density produces ion evaporation [33]. These gas phase ions are then extracted by electrostatic force into the sampling orifice of the transfer capillary [8, 34-36].

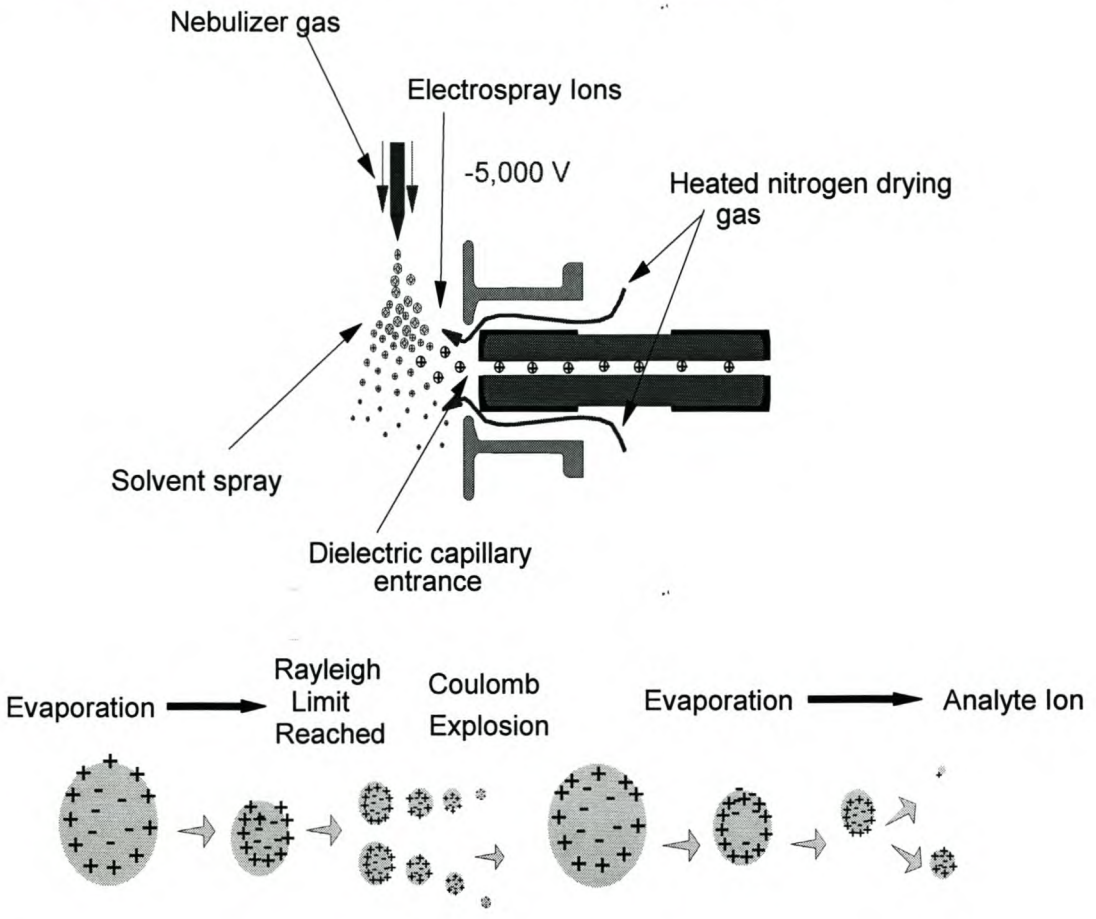

Figure 3.5: A schematic drawing of the electrospray process.

In the APCI process, the first step is the nebulization of the column effluent, followed by evaporation of the droplets. Nebulization in this case occurs within a vaporisation chamber, which is kept at elevated temperature (typically 250-500°C) to assist in the evaporation of the nebulized droplets (figure 3.6). A corona discharge needle is then used to ionise the resultant gas phase analyte molecules. This occurs in a process similar to that used in chemical ionisation in GC, with charge transfer occurring from ionised solvent reagent

molecules to analyte molecules. The charged analyte molecules are then electrostatically extracted to the transfer capillary [8, 33].

The ion optics of a typical LC-MS system consist of a dielectric (transfer) capillary, skimmers, octopole, lenses, quadrupole (or ion-trap) and ion detector (figure 3.6). The dielectric capillary connects the atmospheric pressure ionisation chamber with the low-pressure ion optics region. Charged analyte molecules are extracted from the ionisation chamber by a combination of electrostatic force and the pressure differential. At the outlet of the capillary, the application of a voltage (100-200V) allows for fragmentation of molecular ions using collision-induced dissociation (CID). The skimmers and octopole are used to focus the ion beam. The quadrupole consists of 4 parallel rods, with opposing ones being electrically connected to radio frequency (RF) and direct current (DC) voltages. For any given RF/DC ratio only ions of a specific m/z value are conveyed through the quadrupole to the detector, typically a dynode-based electron multiplier [37].

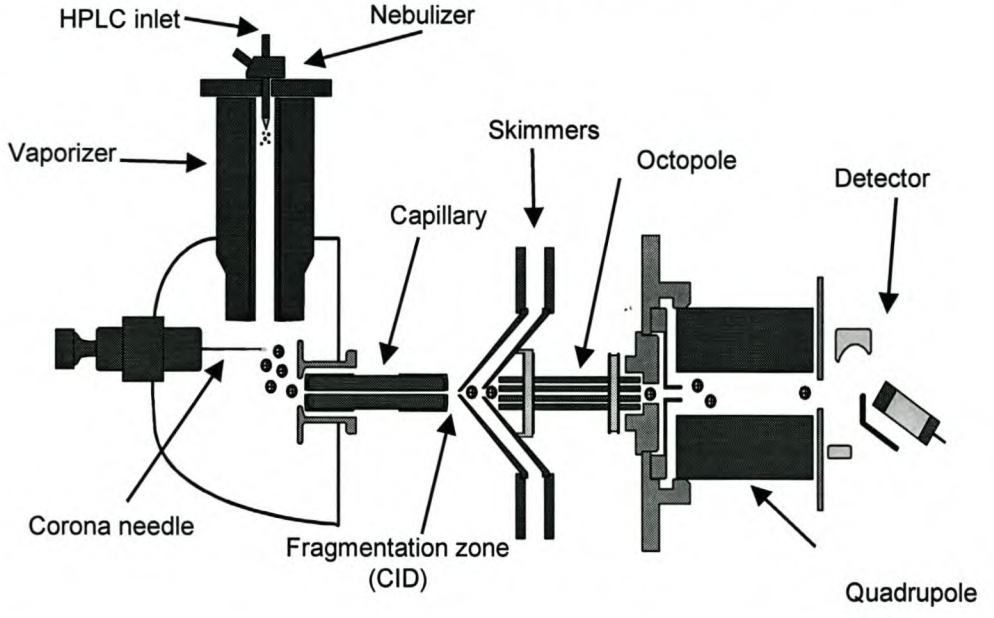

Figure 3.6: The instrumental set-up for APCI, including the ion optics.

Although quadrupole instruments are by far the most commonly used, ion-trap mass analyzers have proved popular in LC-MS applications over the last few years [38]. Ion-trap instruments are based on the same principle as used in quadrupole mass analyzers, with the

difference that the RF controlling the scanning is applied to a circular ring electrode, situated between two end-caps held at ground potential (figure 3.7). Analyte ions are stored in the ion trap by the application of the radio frequency. Scanning is performed by stepwise increments in the RF voltage, causing ions of lower m/z values to become unstable in their orbits and to be expelled towards the detector (electron multiplier) [35, 37]. A major advantage of the ion-trap analyzer over the quadrupole instrument is the ease with which $MS^{(n)}$ experiments can be performed. In this case, the radio frequency is scanned so that only the ion with m/z value of choice remains in the trap. This is followed by CID and scanning of the fragmentation products formed, or by repetition of the same process to provide further fragmentation information.

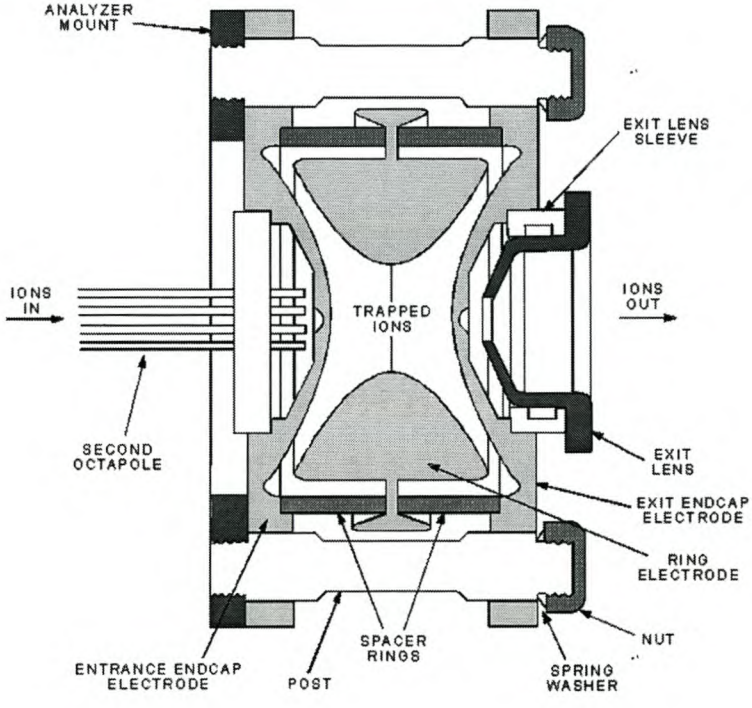

Figure 3.7: Schematic diagram of an ion-trap mass analyzer.

3.3 Capillary Electrophoresis

3.3.1 Principles of Capillary Electrophoresis

3.3.1.1 Introduction

Electrophoresis can be defined as the differential migration of charged species in solution with the application of an electric field (from the Greek words *elektron* = electron, and *phoresis* = carrying). Tiselius introduced electrophoresis as a separation technique in 1937 [39], but since thermal diffusion and convection in large diameter columns limit the efficiency in free solution, electrophoresis has traditionally been performed on glass plates coated with anti-convective media such as polyacrylamide or agarose gels. Although a widely used technique, especially in biochemical fields, slab gel electrophoresis suffers from the drawbacks of long analysis times (because the magnitude of the applied electric field is limited), low efficiencies and difficulties in detection and automation. An alternative to the gel format is to perform the separation in narrow-bore capillaries, since these are themselves anti-convective. Hjertén described initial work in open tube electrophoresis in 1967 [40], but it wasn't until Jorgenson and Lukacs used 75 μm fused silica capillaries in the early 1980's [41] that the theory was fully developed, and that the potential for high performance capillary electrophoresis (HPCE) as an analytical technique was demonstrated.

Separation of solutes in an electric field is the result of their different velocities (v) in the presence of that field:

$$v = \mu_e E \qquad (5)$$

where μ_e is the electrophoretic mobility, and E the applied electric field. The electric field is a function of the applied voltage and the capillary length, while the mobility of an ion in a given medium, is a constant for that ion. The mobility is determined by the electric force experienced by the molecule, balanced by its frictional drag through the medium. The mobility of a spherical ion can be calculated using the following simplified equation, in which the two forces are taken into account:

$$\mu_e = \frac{q}{6\pi\eta r} \qquad (6)$$

where q is the ion charge, η the viscosity of the solution and r the ion radius. From this equation it is evident that smaller, highly charged ions have the largest electrophoretic mobility. The electrophoretic mobility determined experimentally is dependent on the pH of the background electrolyte and thus the solute's pKa, and as such often differs from the values found in standard tables in which values are determined at the point of full solute charge and infinite dilution. This means that it is possible, by choosing the correct pH, to separate solutes with the same mobilities if their pKa values differ.

3.3.1.2 Electroosmotic Flow

Electroosmotic flow (EOF) is the second fundamental constituent of HPCE and is a consequence of the surface charge on the capillary wall and the effect of the applied voltage on the solution double layer at the wall [42]. Under aqueous conditions the fused silica surface possesses an excess of negative charge because of deprotonation of the acidic silanol groups. Counter-ions build up a double layer near these charged surfaces to maintain a charge balance, and thus create a potential difference very close to the wall known as the zeta potential. In the presence of an applied voltage, the cations forming this diffuse double layer are attracted to the cathode, and their solvation causes them to drag the bulk solution in the capillary in this direction (figure 3.8).

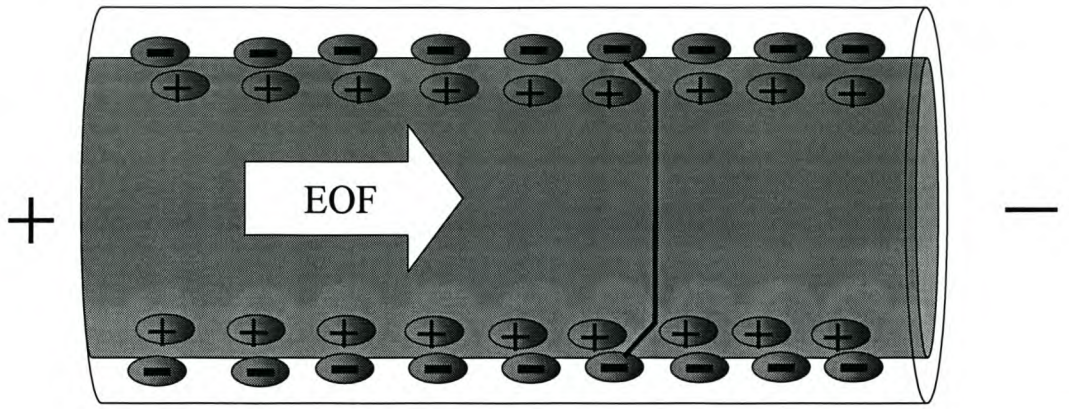

Figure 3.8: Schematic illustration of the generation of the EOF. The negative charges represent deprotonated silanol functions on the capillary wall, the positive charges solvated counter-ions.

The magnitude of the EOF can be described in terms of velocity or mobility:

$$v_{eof} = \left(\frac{\varepsilon\zeta}{\eta}\right)E \text{, or} \qquad (7)$$

$$\mu_{eof} = \left(\frac{\varepsilon\zeta}{\eta}\right) \qquad (8)$$

where ζ is the zeta potential and ε the dielectric constant. In the presence of the EOF the measured mobility is in fact the apparent mobility (μ_a), which can be calculated using experimental parameters:

$$\mu_a = \mu_e + \mu_{eof} = \frac{lL}{tV} \qquad (9)$$

where l is the effective capillary length to the detector, L the total capillary length, V the applied voltage and t the migration time. The electrophoretic mobility can be calculated from the apparent mobility by using a neutral marker such as mesityl oxide or acetone to calculate μ_{eof}.

As a consequence of the fact that the magnitude of the EOF can be more than an order of magnitude greater than the electrophoretic mobilities of the solutes, it is possible to elute

cations, anions and neutrals in one run, with the cations eluting first, the un-separated neutrals with the electroosmotic flow and the anions last. The EOF is, however, not always greater than the mobility of the solute. For small ions, and in cases where the wall charge is decreased while leaving the electrophoretic mobility unaffected, cations and anions will migrate in opposite directions.

Control of the EOF is vitally important in CE separations, and the manipulation of this parameter to suit the separation problem at hand can be achieved in several ways. Since the surface charge of the wall determines the zeta potential, the magnitude of the EOF can be varied by adjusting the pH. The EOF becomes significant at a pH above 3, and increases with pH up to ~9, where the value remains constant, indicating complete dissociation of the silanol groups [43]. In the same way the zeta potential is dependent on the ionic strength of the medium. Increased ionic strength causes double layer compression that results in a decreased zeta potential and EOF. The magnitude of the EOF is directly proportional to the applied voltage, and can be manipulated using this parameter. However, this approach is limited on the one hand by generation of Joule heat, and on the other hand by loss of efficiency (see below). The magnitude, or even direction, of the EOF can be altered through dynamic or permanent coating of the capillary wall [44-46]. For example, the addition of long-chain alkyl ammonium salts to the buffer results in a reversed EOF [47]. Another approach is the addition of organic solvents to the separation buffer, normally leading to a decrease in the EOF [48].

3.3.1.3 Efficiency in Capillary Electrophoresis

The EOF displays a flat flow velocity profile over the cross-section of the capillary [49]. Although the flow rate decreases at the wall, it is relatively unimportant to the overall separation process. This is in contrast to the flow profile generated by pressure such as used in HPLC, where laminar flow yields a parabolic flow profile (figure 3.9).

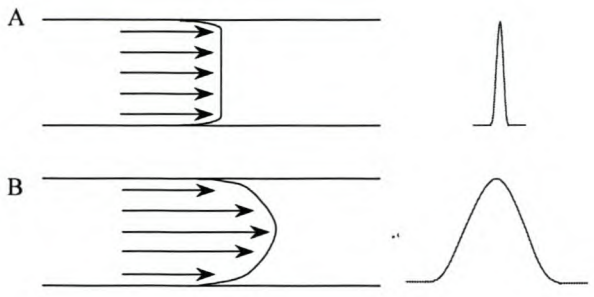

Figure 3.9: Comparison between the flow profiles and corresponding solute dispersion in CE (A) and in HPLC (B).

This flat profile is beneficial since it does not directly contribute to dispersion or solute zone broadening, and therefore reduces the differences in solute mobility needed to separate them. Under ideal conditions (that is, small injection plug length, no solute-wall interactions and in the absence of Joule heating and conductivity perturbations) the sole contributor to zone broadening is longitudinal diffusion. This means that the efficiency can be related to the molecular diffusion term in liquid chromatography (equation 2):

$$H = \frac{\sigma^2}{l} = \frac{2DL}{\mu_e V} = \frac{2D}{\mu_e E} \qquad \textbf{(10)}$$

where σ^2 is the variance of the peak and D is the diffusion coefficient of the solute in the electrolyte medium (D_M). The electrophoretic expression for plate number can now be obtained with:

$$N = \frac{l^2}{\sigma^2} = \frac{\mu_e E l}{2D} \qquad \textbf{(11)}$$

This equation explains why high fields are needed for high efficiencies. The equation also predicts that larger molecules, which have low diffusion coefficients, exhibit less dispersion than small molecules and thus their analysis generally produce higher plate numbers. In practice, however, the measured efficiency is usually lower than the calculated value. The reason for this is that longitudinal diffusion is in practice not the only dispersive process present. Although narrow bore capillaries were originally introduced to reduce the effects of Joule heating (the heat generated by the passage of an electrical current), which has

traditionally limited electrophoretic techniques, Joule heating ultimately limits the magnitude of the electric field which can be applied. Heating causes non-uniform radial temperature gradients, which results in local changes in viscosity and concomitant zone broadening. To reduce the effects of Joule heating, it is best to use very narrow bore capillaries so that less heat is generated. The high inner-surface to volume ratio helps to dissipate the generated heat faster. Additionally, the buffer ionic strength should be decreased to limit Joule heating. However, this decreases the buffering capacity and may increase the potential for solute-wall interactions. Active temperature control is crucial, not only in dissipating the heat, but also to keep the temperature of the capillary lumen stable. This is because a change of 1°C in temperature can result in viscosity (and thus mobility) changes of 2-3 % [50]. This alters not only the EOF and the velocities of the solutes, but also causes fluctuations in injected volumes, thereby detrimentally affecting the reproducibility.

3.3.1.4 Resolution in Capillary Electrophoresis

The resolution in HPCE is expressed by:

$$R = \left(\frac{1}{4\sqrt{2}}\right)(\Delta\mu)\left(\frac{V}{D(\overline{\mu} + \mu_{EOF})}\right)^{1/2} \qquad \textbf{(12)}$$

where $\Delta\mu = \mu_{e2} - \mu_{e1}$, and $\overline{\mu} = (\mu_{e2} + \mu_{e1}) / 2$. It is evident that an increase in the applied voltage does not result in a linear increase in resolution as it does in the case of efficiency because of the square root relationship. Thus very high voltage increases are required to increase the resolution by a substantial amount, although, once again, this approach is often limited by Joule heating.

3.3.2 Modes of Operation

The key to the versatility of CE lies in the numerous operational modes of the technique, each of which relies on a different separation mechanism and thus has a different selectivity. The various modes will be discussed briefly.

3.3.2.1 Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE) is the most widely used mode, mainly because of its simplicity and versatility. In this form, separation occurs in a capillary filled with buffer or background electrolyte (BGE), and ideally discrete solute zones will possess different velocities as a consequence of the interplay between their electrophoretic mobilities and electroosmosis. Neutral molecules are not separated in this mode and elute with the EOF.
Of primary importance for a successful CZE separation is the choice of running buffer. The following requirements are important to consider when choosing a buffer:

- Good buffering capacity in the pH range of choice,
- Low absorbance at the wavelength of detection
- Low mobility to minimise current generation
- The conductivity of the buffer should match that of the sample solvent as closely as possible to reduce peak shape distortion.

One of the most attractive features of CE, in general, is the number of options available to the analyst to tune the separation selectivity. This is also true for CZE, where the first step is normally the optimisation of the pH. This parameter has to be chosen with care since it affects not only the charge of the solute molecules, but also the magnitude of the EOF, and can influence possible interactions between solutes and the capillary wall. Modification of capillary surfaces is performed to manipulate the EOF and to suppress analyte-wall interactions. Thus, by dynamically or permanently coating the wall with positive, neutral or negative coatings it is possible to reverse, nullify or increase the EOF [44]. Chiral separations are possible by simple addition of a chiral selector (e.g. neutral or charged cyclodextrins, crown ethers or bile salts) to the buffer. Other buffer additives can improve

separation through preferential complexation of specific analytes, enhancing differences in electrophoretic mobilities [51, 52].

3.3.2.2 Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC) is a hybrid of electrophoresis and chromatography, and was introduced by Terabe in 1984 [53]. It has become one of the most widely used CE modes because of the possibility it offers to separate neutral molecules. This is accomplished by adding surfactants to the running buffer. Above the critical micelle concentration (CMC), micelles are formed, and these can migrate either with or against the EOF, depending on the charge. During their migration, micelles interact with solute molecules in a chromatographic manner through hydrophobic interactions. In the case of anionic surfactants such as sodium dodecyl sulphate (SDS), the micelles migrate in the opposite direction to the EOF (to the anode). Since the EOF is faster than the electrophoretic velocity of the micelles, the net migration is in the direction of the EOF. Neutral molecules are separated as a result of differential partitioning between the micelles and the aqueous buffer, determined by their relative hydrophobicity. The more hydrophobic molecules are thus retained longer and elute later. Since the separation mechanism of neutral molecules in MEKC is essentially chromatographic, the capacity factor k (the ratio of the moles of solute in the micelles to those in the mobile phase), has to be taken into account with respect to a moving stationary phase (pseudostationary), and can be derived by:

$$k = \frac{(t_r - t_0)}{t_0\left(1 - \frac{t_r}{t_m}\right)} = K\left(\frac{V_S}{V_M}\right) \qquad \textbf{(13)}$$

where t_r is the migration time of the solute, t_0 the migration time of an unretained solute or EOF marker, t_m the micelle migration time, K the distribution coefficient, V_s the volume of micellar phase and V_m the volume of mobile phase or electrolyte. In general the capacity factor increases linearly with surfactant concentration, although in the case of ionic surfactants the resulting increase in current often limits the amount of surfactant which can

be added. All neutral molecules elute in the time window between t_0 and t_m. It is therefore desirable to extend this window. This can be done by slowing down the EOF and using micelles with a high mobility. Selectivity in MEKC is easily adjusted by varying the physical nature of the micelles by using different surfactants. In every case the buffer concentration, pH, and temperature can also be adjusted to optimise selectivity. Addition of ionic species (*i.e.* electrolytes) will result in a decrease in the CMC and a slight increase in the aggregation numbers (the number of surfactant molecules comprising the micellar entity). The choice of surfactant determines the selectivity of the MEKC separation, and various alternatives to the long-chain alkyl surfactants commonly used have been proposed [54]. In addition, organic modifiers such as methanol or acetonitrile can be added to the buffer to modify solute-micelle interaction. For example, short-chain alcohols can enhance micelle formation (in other words reduce the CMC) when present at low concentration, but prevent micelle formation at higher concentrations, while organic solvents like acetone or acetonitrile will slightly inhibit micelle formation at low concentration and prevent it at higher concentrations [54]. When a hydrophobic organic solvent is added to a micellar system, together with a slightly more hydrophilic solvent for stabilisation purposes, a microemulsion is formed. Microemulsions can be utilised as running buffers in capillary electrophoresis, in a method referred to as microemulsion electrokinetic chromatography (MEEKC) [55]. Penetration of the solvent into the micellar phase is thought to result in a reduction of the surface charge on micelles [56]. Differences in elution characteristics between MEKC and MEEKC have been reported, suggesting the use of MEEKC as an approach to alter selectivity of MEKC separations [56, 57].

3.3.2.3 Capillary Gel Electrophoresis

Slab gel electrophoresis has been used primarily in the biological sciences for the separation of macromolecules such as proteins, DNA fragments and polysaccharides. This is achieved by electrophoresis through a suitable polymer that acts as a molecular sieve by hindering larger solutes more than smaller ones during their migration. The CE format of slab gel electrophoresis, capillary gel electrophoresis (CGE), offers certain advantages over the traditional slab gel electrophoresis, which can sometimes be a laborious and time-

consuming technique. Advantages include the use of higher field strengths without the detrimental effect of Joule heating, on-capillary detection and automation (although DNA-sequencers are commercially available). The use of anti-convective gels such as agarose or polyacrylamide to eliminate conductive transport and reduce diffusion is a necessity for slab gel electrophoresis and places limits on the choice and concentration of material used. The anti-convective nature of capillaries relaxes these limitations. Since the publication of the first papers on the use of CGE in the early 1980s [58] it has become evident that almost all methods developed for slab gel electrophoresis can easily be transferred to the capillary format, with the concomitant advantages of high resolution, automation and on-line data acquisition and storage. The multi-lane capabilities of the slab gel format can be matched in CGE using systems employing multiple capillaries (for example in DNA sequencing), although rapid analysis times in the case of CGE do not compensate for the preparative capabilities of the slab gel format [59]. Since CGE is also a "zonal" electrophoretic technique, the resolution and efficiency obtained are often higher than those obtained by CZE and MEKC. Selectivity in CGE can also be altered by varying the physical characteristics of the polymer used, or by simple addition of chiral selectors and complexing agents to the running buffer.

3.3.2.4 Capillary Isoelectric Focussing

Similar to CGE, capillary isoelectric focussing (CIEF) was adapted to the capillary format from a well-established gel electrophoretic technique. CIEF is mainly used to separate proteins and peptides based on pI values. In this technique ampholytes are used to create a pH gradient within the capillary, with a basic solution at the cathode and an acidic solution at the anode. Application of an electric field leads to the migration of proteins and ampholytes to the point in the capillary where they are not charged (at the pI value). During focussing, the EOF is reduced or eliminated by covalent or permanent coating of the wall, in order to avoid flushing of the ampholytes from the capillary [60]. When the focussing process is completed (as indicated by the loss of current), the various zones are mobilized through the detector, normally by pressure [61].

3.3.2.5 Capillary Isotachophoresis

Capillary isotachophoresis (CITP) is based on the use of leading and terminating electrolytes, each with mobility higher and lower than those of the analytes, respectively. Under these conditions the ions migrate in discrete zones, with identical velocities, as determined by the leading electrolyte. Since there is a constant concentration in each zone, as determined by the leading electrolyte, sharpening of less concentrated zones occurs. Isotachophoretic principles are therefore often used in other forms of CE as a pre-concentration step [62]. Drawbacks of the method include the fact that only anions or cations can be analysed in a single run, while choice of suitable leading and terminating electrolytes is often problematic [61].

3.3.3 Instrumental Aspects of Capillary Electrophoresis

The basic instrumental set-up of capillary electrophoresis instrumentation is depicted in figure 3.10. Each aspect will be discussed shortly. Fused silica capillaries are the most commonly used columns in CE, ranging in internal diameters from 10 to 200 μm, and in total lengths from 10 to 120 cm. The silica is coated with a layer of polyimide to make them more durable and practical. A small section of this coating is removed for detection purposes. Fused silica capillaries coated with a variety of materials are commercially available for specific applications where the magnitude of the EOF is tuned for specific applications.

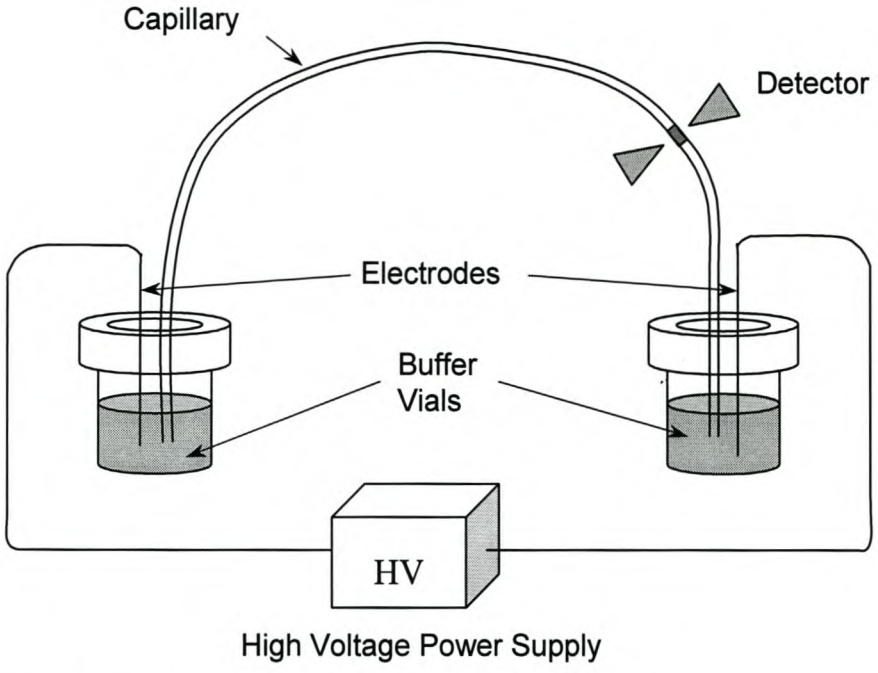

Figure 3.10: The basic constituents of a CE instrument.

Because of the strong dependence of viscosity on the temperature, effective capillary thermostating is essential. This can be achieved by liquid- or air-cooling of the capillary in a closed environment such as a capillary cassette, with the liquid-cooling method providing more reliable results. A DC power supply capable of applying up to 30 kV and current levels of up to 300 μA is used in CE instruments. The power supply is capable of switching the applied polarity for use in cases where the EOF is reduced or reversed. In addition, these power supplies have the ability to run voltage, current or power gradients (called field programming). Vials are placed in one or two trays. Automated control allows replacement of the inlet buffer vial by sample vials for injection, flush vials, or vials containing rinsing buffer. Hence, analogues to HPLC, modern CE instrumentation is generally fully automated, allowing sequential analysis with all parameters such as injection-and flushing cylces, etc. to be specified prior to analysis.

3.3.3.1 Injection Modes in Capillary Electrophoresis

There are two methods of sample introduction in CE: hydrodynamic injection and electrokinetic injection. The former is based on pressure differences between the inlet and outlet ends of the capillary, which can be produced using gravity, overpressure or vacuum mechanisms. In gravimetric or siphonic injection the sample vial at the inlet of the capillary is raised to a predetermined height for a defined time, and the resultant hydrostatic pressure forces the sample into the capillary. Alternatively, pressure can be applied to the sample vial on the inlet end of the capillary, or vacuum can be applied to the outlet to suck the sample into the capillary. This form of injection is universally applicable to all sample matrices and displays no bias toward sample components. In general pressure injection delivers better reproducibility and greater control over the amount of sample injected compared to electrokinetic injection.

During electrokinetic injection, the sample vial is placed on the inlet side of the capillary and high voltage is applied for a short period of time. The solute molecules migrate into the capillary due to a combination of electrophoretic migration of the ions and electroosmotic flow of the sample solution. This means that a difference in electrophoretic mobility between different solutes translates into a bias in injection amount between these solutes. Reproducible electrokinetic injection is, however, possible at much smaller volumes than in the case of hydrodynamic injection. Because the apparatus needed for electrokinetic injection has basically the same arrangement as the separation process, the ease of operation sometimes makes this the preferred form of injection.

In CE techniques, sample plugs are commonly very small (nanoliters). This results in low sensitivity. Various methods to increase the sensitivity in CE have been proposed. Sample stacking is a method used to increase the sensitivity of CE analyses, where the aim is to convert a large volume of a low concentration analyte into a sharp zone of higher concentration. Two main approaches can be distinguished: isotachophoresis [63] (discussed above), and field amplified sample stacking. In the latter technique, differences in sample zone and running buffer conductivities are exploited.

The correlation between the electric field strength (E), the current density (i), and the conductivity (κ) of the electrolyte can be expressed as:

$$E = \frac{i}{\kappa} \qquad \textbf{(14)}$$

It follows that ions in a low-conductivity sample zone, injected into a capillary filled with high conductivity buffer, will experience greater field strength in the sample zone than in the buffer. These ions will accelerate to the concentration boundary of the buffer, where they will slow down and be stacked into a narrow zone, in a process referred to as stacking (figure 3.11). Positive ions stack at the front of the sample zone, and negative ions at the back, while neutral molecules will remain unaffected. Subsequently, the reduced ionic zones move into the buffer region and electrophoresis will occur in the normal manner.

The amount of stacking is theoretically only proportional to the field enhancement factor ($=E_{sample}/E_{buffer}$), suggesting the opportunity to stack a very large, diluted sample more efficiently. There are, however, limitations with regard to the concentration differences between the buffers to be used and the length of the sample plug to be injected. This is because, as the conductivity of the sample zone decreases, the electric field in the supporting buffer approaches zero, negating any chance of separation, and the unseparated analytes will then be pushed from the capillary by the EOF. In addition, zone broadening occurs as a consequence of laminar flow resulting from mismatches between local electroosmotic velocities and the bulk velocity. Thus there is an optimal point as to the injection plug length and the conductivity differences to be used [64].

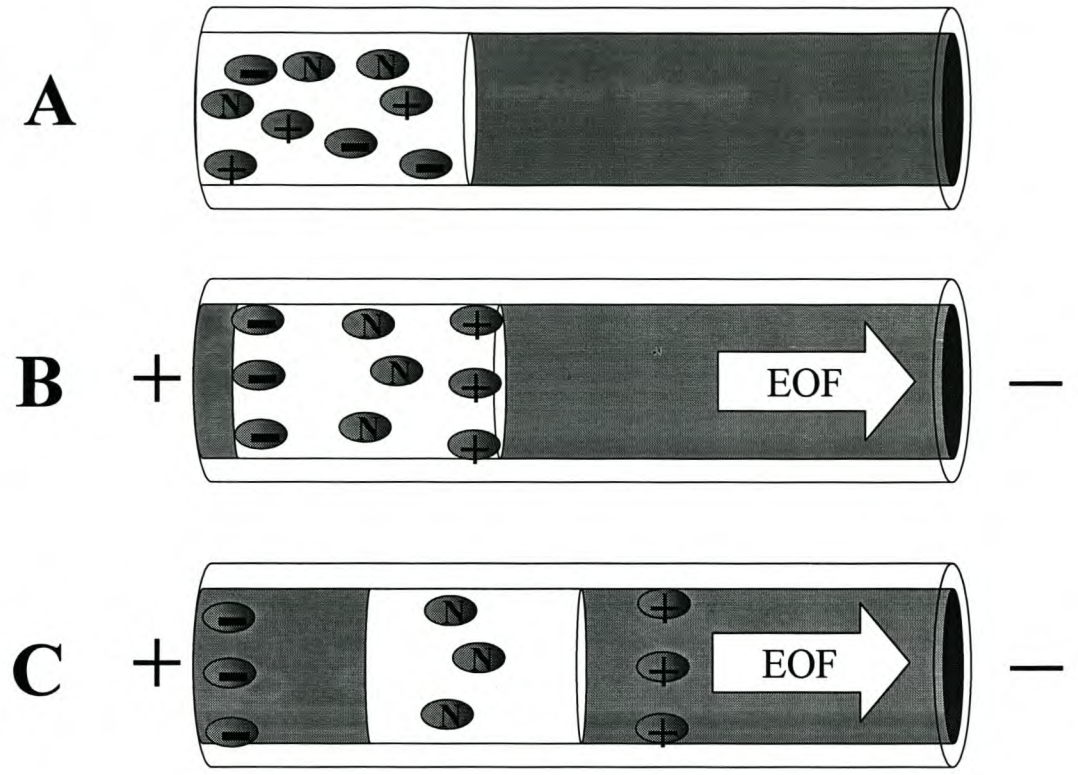

Figure 3.11: Sample stacking. In step A a large plug of the sample dissolved in a low conductivity matrix is injected hydrodynamically. In step B, with the application of the separation voltage, the ionic solutes stack at the boundaries of the sample plug because they experience a lower field when crossing the concentration boundary. In step C the sample plug is removed by the EOF and electrophoresis continues normally.

This situation can be improved by removing the excess sample solvent. In an elegant solution proposed by Burgi and Chien [65], this was achieved by reversal of the applied voltage, thereby using the EOF to remove the bulk of the sample solvent. In this case negative ions stack at the front of the sample plug, and once most of the solvent has been removed (as indicated by the current approaching the normal level in a buffer-filled capillary), the voltage is reversed and electrophoresis proceeds in the normal manner. It has to be noted that only specific ions (i.e. positive or negative) can be stacked in this manner. Related methods have been used for on-line concentration of various samples [66], particularly in cases where interfering sample components present in excess have to be

removed prior to analysis [67-69]. The addition of organic solvents to the sample matrix to reduce the conductivity and improve stacking efficiency has also been demonstrated [68, 70]. The methodology is not only limited to CZE, and the applicability of related methods has been demonstrated for neutral [71, 72] and anionic [73, 74] solutes in MEKC separations.

An additional form of on-line sample pre-concentration, called dynamic pH junction, is based on the difference in degree of ionisation of the analyte between the buffer and sample zones. For example, by choosing the pH of sample and buffer regions carefully, it is possible to stack weakly acidic compounds from a low pH sample plug to a high pH buffer [75, 76], or basic compounds from a low pH sample plug to a higher pH buffer [77]. The merit of these methods lies in their simplicity, requiring no additional instrumental features or sample preparation, apart from dilution or pH adjustment.

3.3.3.2 Detection

Detection in CE poses a challenge due to the high efficiencies, small peak volumes and limited time available for detection. Many of the common detection methods used in HPLC have been adapted for use in CE. The principles of both UV and MS detection have been discussed previously. Only those aspects relevant to their application in CE will be outlined briefly.

3.3.3.2.1 UV Detection

This is the most common form of detection used in CE applications, and most commercial instruments are equipped with an UV detector. On-column UV detection is performed simply by removing a small section of the polyimide coating of the capillary to create a UV-transparent window. Since the window is situated on the capillary, there is no real post-column zone broadening as a result of dead volume. However, a too large window size with respect to the length of the eluting peak can cause an optical band broadening of the zone. Because of the short period of time analytes spend in the detector (as short as 1-2s), the detector response times must often be faster than for other separation methods.

background electrolyte contains a UV-absorbing co-ion (i.e. of the same charge), referred to as the visualizing ion. Displacement of the visualizing ion by the analyte leads to a drop in the background UV signal, and a negative peak is detected when the sample zone passes the detector. The use of UV absorbing counter-ions has been reported [80], although this approach is uncommon, leading to poor sensitivity (see below).

It follows that all ions can be detected in this manner. The sensitivity is determined by the transfer ratio, which specifies the degree of displacement of BGE ions. The transfer ratios (TR) in the case of a strong ion *i* in a BGE consisting of strong ions *vis* (the visualizing ion) and *count* (the BGE counter-ion), are given by [81]:

$$TR_{i,vis} = -\frac{z_i}{z_{vis}} \times \frac{\mu_{vis}(\mu_i - \mu_{count})}{(\mu_{count} - \mu_{vis})\mu_i} \quad \textbf{(15)}$$

$$TR_{i,count} = \frac{z_i}{z_{count}} \times \frac{\mu_{count}(\mu_i - \mu_{vis})}{(\mu_{count} - \mu_{vis})\mu_i} \quad \textbf{(16)}$$

where $TR_{i,vis}$ and $TR_{i,count}$ are the transfer ratios for the visualizing- and counter ions respectively, and μ_i, μ_{vis} and μ_{count} are the mobilities of the ion *i*, the visualizing ion and the counter ion, respectively. In the simplest form, where $\mu_i = \mu_{vis}$, $TR_{i,vis}$ is dependant only on the ratio of the solute charge to that of the visualizing ion, while $TR_{i,count}$ is 0. This situation rarely occurs in practice though, as in most CE applications the BGE must function as buffer, and is thus only partially charged, while additional additives lead to multiple displacement reactions, thereby decreasing $TR_{i,vis}$ [82].

A characteristic feature of CE-IAD is the relatively poor peak shapes found in most applications. A typical electropherogram of a complex sample will contain fronting peaks at the beginning, followed by some symmetrical peaks, while the last peaks will display tailing. This is the result of electrodispersion – the process where differences in mobility between analyte and buffer ions lead to dissimilar field strengths (E) in their respective zones and concomitant distorted peak shapes. This distortion is proportional to the mobility differences between the analyte and the buffer, with identical mobilities resulting in zero distortion. This phenomenon becomes significant only when the analyte concentration approaches that of the BGE, a situation prevalent in CE-IAD, since the low BGE concentrations are required to provide a sufficiently sensitive and linear response [81].

Another aspect of CE-IAD is the detection of disturbances in the BGE, the so-called system zones [83]. These zones are detected when working with a visualizing ion in the BGE, and may interfere with the separation.

3.3.3.2.2 Mass Spectroscopy

The coupling of CE to MS is a relatively new development in analytical chemistry [84], and the technique is in full development. A recent overview of the current state of CE-MS developments can be found in [85]. Electrospray (ESI) ionisation is mostly used in CE-MS, with instrumentation often consisting of LC-ESI-MS equipment modified to accommodate the peculiarities of CE. The primary adjustment to be made is the addition of a make-up or sheath liquid to augment the nL/min CE flow to the level of ~10 µl/min in order to allow successful electrospray ionisation. The introduction of the sheath liquid allows post-column modification of the solution chemistry to enhance ESI ionisation, but at the same time this leads to unwanted band broadening and analyte dilution. Generally, the sheath liquid consists of mixtures of organic solvents such as methanol and isopropanol together with volatile buffers to improve ionisation. The sheath-flow capillary is made of stainless steel to provide electrical contact.

The coupling to MS does limit the choice of buffer to be used for the CE separation. Volatile buffers (e.g. acetic, formic acid, triethylamine) are most suited to this form of detection, but are limited in the useful pH range, while often leading to reduced separation efficiency [86]. Limited amounts of the common non-volatile CE buffers such as phosphate and borate can be tolerated. Notwithstanding the sensitivity of the MS, one of the major limitations of CE-MS remains the small sample capacity of CE. This leads to a high concentration detection limit even though the absolute amount detected can be quite low. A variety of solutions involving developments on both the CE and MS ends have been suggested to overcome this problem. These include methods for loading higher sample amounts from dilute solutions such as transient isotachophoresis (tITP) and membrane pre-concentration, as well as new developments in MS detectors and interfaces [87].

3.4 Sample Preparation

Despite the array of sophisticated analytical methods available today, direct injection of samples is often not possible. Samples may be too complex, or analytes present in too low amounts, or the sample may simply be incompatible with the analytical method to be used. Thus it is no surprise that a vast number of (often complex) sample preparation methodologies, based on physical, chemical or chromatographic principles, have been developed to allow the efficient extraction and clean-up of analytes from a variety of different matrices. The following brief discussion will be limited to those forms of sample preparation utilized in this study.

3.4.1 Liquid-Liquid Extraction

This is a form of solvent extraction where two immiscible liquids are placed in contact, and partitioning of solutes between the two phases takes place. This partitioning is the result of differential affinity of each solute for the two phases involved, and is quantitavely described by the distribution coefficient K_D ($=[solute]_{phase1}/[solute]_{phase2}$). Various liquid-liquid extraction (LLE) procedures have been developed together with dedicated apparatus suitable for each [88]. In its simplest form, LLE is performed in a separation funnel, where the two phases are shaken together before being allowed to separate. In cases where the distribution coefficients are unfavourable, multiple extractions are often performed. Exhaustive extractions of especially solid samples may be performed using a Soxhlet extractor, where the extracting solvent is automatically distilled, condensed and passed through the sample. Following extraction, the solvent can be removed by evaporation, and the residue dissolved in a matrix suitable for analysis. This also allows pre-concentration of the sample. LLE is still regularly used in analytical procedures, mainly because of the simplicity and the familiarity of the method (a large number of LLE procedures may be found in the literature). Drawbacks include the use of large amounts of expensive, and often toxic, solvents, the manual and time-consuming nature of the procedure, and the formation of emulsions.

3.4.2 Solid Phase Extraction

Solid phase extraction (SPE) was developed in the 1970s as an alternative to liquid-liquid extraction. In SPE analytes are purified and/or pre-concentrated by sorption onto a disposable cartridge, followed by rinsing and elution with appropriate solvents. Retention of analytes is based on the same principles as discussed for HPLC: reversed- and normal phase partitioning and ion exchange. The generic SPE procedure consists of four steps, namely conditioning, sample application, rinsing, and analyte elution. The aim of the conditioning step is to activate the packing material for effective sorption. For aqueous samples, a common procedure is to use methanol followed by water. During sample loading it is important to ensure that the retention mechanism functions effectively (i.e. under conditions of the sample solvent). Next, the column can be rinsed with a suitable solvent in order to remove sample components not of interest, if needed. Finally, the analytes of interest are removed using a (preferably small) volume of a solvent capable of disrupting the analyte-sorbent interaction, while additional interferents will ideally remain on the cartridge. An alternative approach is to rinse the analytes from the cartridge in the second step, while other sample components remain sorbed on the cartridge [89].

Typical SPE cartridges contain between 50 mg and 1 g of 40 μm (low efficiency) packing material in a disposable plastic column, and can handle between 1 and 1000 mL of sample. Elution of samples and solvents can be achieved using positive pressure or vacuum. Because relatively large sample volumes can be loaded onto small packed beds, and elution can often be preformed using minimal solvent volumes, significant pre-concentration can be achieved, often without involving a solvent evaporation step.

The versatility and speed of SPE methods have primarily been responsible for the widespread popularity of these methods. Flexibility stems from the fact that many established LC approaches can be used to modify the selectivity in SPE. High recoveries and low solvent consumption also add to the attractiveness of SPE, specifically compared to LLE. The method lends itself to automation, and in fact automated SPE stations are

commercially available. Finally, coupling of automated SPE procedures to LC and GC allows on-line sample preparation for various applications [90].

3.4.3 Stir Bar Sorptive Extraction

Stir bar sorptive extraction (SBSE) is a new solventless extraction method where solutes are enriched from (normally aqueous) sample matrices by absorption into a polydimethyl-siloxane (PDMS) -coated stir bar [91]. Numerous methods, based on the use of PDMS phases, have been described for the extraction of analytes from liquid and gaseous samples [92]. For aqueous samples the analytes are partitioned between the PDMS- and water phase based on their octanol-water coefficients and the phase ratio ($=volume_{aqueous}/volume_{PDMS}$) [93]. Compared to the other popular absorptive sample extraction procedure, solid phase microextraction (SPME), SBSE offers a relatively large amount of stationary phase, allowing higher recoveries and a higher sample capacity [94].

Initial applications of SBSE focused on the analysis of volatiles and semi-volatiles in combination with thermal desorption (TD)-capillary GC (cGC). Thus this procedure has proved highly suitable for the analysis these compounds in water [95], beverages [96] and even fruit [97]. More recently though, the combination of SBSE with liquid desorption (LD) has been investigated for the analysis of thermally labile compounds. In combination with LC analysis, SBSE-LD has been applied to the analysis of polyaromatic hydrocarbons (PAH) [98] and fungicides [99]. In this instance, following sorption by stirring in the sample matrix, the analytes are desorbed by stirring in a small volume of a suitable solvent, which can then be injected for HPLC or CE analysis.

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4

Development of a Solid Phase Extraction Procedure for the Determination of Polyphenols, Organic Acids and Sugars in Wine*

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4.1 Introduction

The development of analytical methods for diverse classes of wine and grape constituents is of great importance to the wine industry. Monitoring of the chemical content of grapes and wines is needed to study the ripening of grapes, the fermentation and ageing processes, as well as to analyse the authenticity of wines. Moreover, increasing demands are being placed on producing wines of constant quality.

A number of non-volatile compounds play an important role in the character of a wine. Organic acids are influential in determining the sensory properties and stability of the wine, while sugars (mainly glucose and fructose) have a direct bearing on the organoleptic profile thereof [1]. The phenolic compounds play an essential role in the sensory properties (some of these compounds are responsible for bitterness, astringency and the colour of red wines) and in ageing [2, 3]. Some of the phenols have been linked to health benefits associated with drinking wine [4, 5].

Exhaustive investigations into the most suitable analysis methods for these diverse compounds have been performed. For the organic acids, the applicability of reversed phase liquid chromatography (RP-LC) [6-9], often requiring derivatization, and recently capillary electrophoresis (CE) [10, 11] have been demonstrated. However, the method of choice in a routine environment, is ion exclusion chromatography (IEC) with UV detection [12-15]. For sugars, LC is commonly used, either in the normal phase [16, 17] or ion-exchange [18-20] mode. Detection is done using refractive index (RI), pulsed amperometric or evaporative light scattering (ELSD) detectors [19, 20]. Although normal phase LC (NP-LC) [21, 22] and CE [23-26] have been applied for the analysis of polyphenols, RP-LC is by far the most powerful analytical method. Detection methods include UV and AP-ESI MS [27-35].

The separation methods mentioned above have shown to be capable of determining most of the compounds of interest. The complexity of the wine sample, however, necessitates the use of some form of sample preparation and/or clean-up. For example, UV detection at 210 nm and RI or ELSD detection are commonly used for the detection of organic acids and sugars,

respectively. With these non-selective modes of detection, direct injection of wine is not feasible. While the option of coupling MS detection to the LC analysis of wines greatly improves the identification power of the method, quantitation of key phenolics, for example, still remains a problem. Moreover LC-MS is not yet considered a routine technique for wine laboratories.

Generally, methods of sample preparation for the LC analysis of organic acids and sugars are based on the use of solid phase extraction (SPE) cartridges, where the hydrophobic wine constituents are retained while the analytes are eluted with an aqueous solution [8, 20]. Similarly, many reports on the use of SPE for sample clean-up prior to polyphenol analysis have been published [27-31, 33, 36]. Compared to liquid-liquid extraction, SPE offers the advantages of increased speed and selectivity, improved recoveries as well as the option of automation. Oszmianski et *al.* demonstrated the fractionation of the phenolic compounds in wine into four groups namely the phenolic acids, the flavanols, the flavonols and the polymeric phenolics) using C_{18} SPE cartridges [28]. While methods based on this work have been shown to be effective in simplifying LC chromatograms, this approach necessitates 2-4 analyses per sample in cases where the analyst is interested in a complete phenolic pattern. Also, quantitative recovery of the different classes of phenolics is impossible, and recoveries vary depending on the wine being analysed. Thus it seems that for the analysis of polyphenols in wine, a compromise has to be made between the simplicity of the chromatogram, and the amount of reliable information one wants to obtain from each analysis. In this chapter, a single step SPE procedure that provides samples suitable for the analysis of organic acids, sugars and monomeric polyphenols is presented. The method was optimised by simulating a wine sample including 27 standard compounds (2 sugars, 7 organic acids and 18 polyphenols).

4.2 Experimental

4.2.1 Materials

Analytical grade standards were purchased from Sigma-Aldrich (Atlasville, South Africa), Riedel-de Haën (Midrand, South Africa), Acros (Geel, Belgium) and Merck (Darmstadt, Germany). HPLC grade acetonitrile was from Sigma, sulphuric acid and ethyl acetate were from Merck. LC mobile phases were filtered through 0.45 µm HV filters before use (Millipore Corporation, Bedford, MA, USA). The styrene-divinylbenzene SPE cartridges (Strata SDB-L, and Chromabond HR-P, each 3 mL, 500 mg phase) were from Phenomenex (Torrance, CA, USA) and Macherey-Nagel (Düren, Germany), respectively. The tC_{18} Sep-Pak Vac (3 mL, 500 mg phase) cartridges were from Waters (Milford, MA). Wine samples were purchased from local stores. If not analysed directly, the samples were transferred under nitrogen to completely filled amber bottles to ensure their preservation.

4.2.2 Instrumentation and Chromatographic Conditions

LC analysis of polyphenols and organic acids were carried out on an Alliance 2690 Separations Module equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). Data analysis was done with Millenium[32] Chromatography Manager software. The sugar analyses were performed on a modular system consisting of a Waters 510 pump equipped with a U6K injector, an evaporative light scattering detector (500 ELSD) from Alltech (Deerfield, IL), and an HP 3396 integrator from Agilent Technologies (Pinelands, South Africa).

For the organic acid analysis an Aminex HPX-87H Ion Exclusion column, 30 cm × 7.8 mm i.d.) and guard column of the same phase were used (Bio-Rad, Nazareth, Belgium). The mobile phase consisted of an aqueous solution of 20 mM H_2SO_4. All experiments were performed at a flow rate of 0.6 $mL \cdot min^{-1}$. The injection volume was 10 µL and the column temperature was kept constant at 50°C. Detection was performed at 210 nm.

A Spherisorb NH_2 column (25 cm × 4.6 mm i.d., 5 µm particles) from Waters was used for the sugar analysis with a mobile phase consisting of 87 % acetonitrile/13% water. The flow rate

was 1.1 $mL \cdot min^{-1}$, and the injection volume 10 µL. The ELSD settings were as follows: nebulizer gas flow 2.74 $L \cdot min^{-1}$ and drift tube temperature 85°C.

Polyphenol analysis was carried out on a Phenomenex Luna C18 column (25 cm × 4.6 mm i.d., 5 µm particles). The mobile phase consisted of (A) 2% acetic acid in water, and (B) 0.5% acetic acid in 50/50 acetonitrile/water. The following gradient was used: 10 to 36% B in 30 min, 36-55% B in 20 min, 55-100% B in 10 min and 100% B for 15 min before returning to the initial conditions. The injection volume was 20 µL and the column temperature 25°C. A flow rate of 1 $mL \cdot min^{-1}$ was used, and detection was performed at 280, 315 and 370 nm, for detection of flavanols, phenolic acids and flavonols, respectively. UV spectra over the range 200-600 nm were recorded. Both the retention time and the UV spectra were used to identify the polyphenols in wine samples.

4.2.3 Procedure for Solid Phase Extraction

The finalised SPE procedure is as follows. The Strata SDB-L cartridges were conditioned with 3 mL each of ethyl acetate, methanol and water (pH 2.5, adjusted with 1 M HCl). The pH of the wine sample was adjusted to 2.5 with 6 M HCl, prior to spiking with 5000 ppm formic acid, the internal standard for the organic acid analysis. 1 mL of this sample was loaded onto the cartridge. The organic acids and sugars were removed with 4 × 1 mL of 20 mM sulphuric acid. This combined eluent (5 mL) is used for both sugar and organic acid analyses. The polyphenols were then eluted with 5 × 2 mL ethyl acetate. The combined eluent was evaporated and re-dissolved in 1 mL 50% acetonitrile/50% water. The final volume of the sample was brought to 2 mL with water prior to injection. Recoveries were studied using an artificial wine sample (13% ethanol) containing each of the 27 standards at a level close to that expected in wine (table 4.1) before and after SPE clean-up. Repeatability's (n=6) refer to the sample preparation and LC procedures.

Quantitation of the solutes in wine samples is performed as follows. Calibration samples containing 100, 500, 1000, 2500 and 5000 $mg \cdot L^{-1}$ (ppm) of the six acids (table 4.1) are

prepared in deionised water and the spiked 5000 ppm formic acid is used as internal standard. External calibration for fructose and glucose is performed using standard solutions in 80 % acetonitrile. The calibration levels are the same as for the organic acids. Calibration samples containing 0.5, 1, 5, 25 and 50 $mg \cdot L^{-1}$ (ppm) of the 18 phenolic standard (table 4.1) is prepared in 40% methanol for external standardisation.

No.	Compound	Amount (ppm)	Average recovery	%rsd
1	Citric acid	1000	95.5	0.9
2	Tartaric acid	2250	100.4	0.6
3	Malic acid	2000	98.0	0.8
4	Succinic acid	1250	81.1	2.2
5	Lactic acid	1350	75.3	2.6
6	Formic acid	5000	90.3	1.4
7	Acetic acid	910	89.9	2.1
8	Fructose	1000	106.5	3.7
9	Glucose	1000	91.1	2.5
10	Gallic acid	29	51.0	3.5
11	Protocatechuic acid	2	92.6	2.5
12	Catechol	34	89.6	2.2
13	Catechin	29	76.3	3.2
14	Chlorogenic acid	0.5	92.2	1.1
15	Vanillic acid	8	97.0	2.3
16	Caffeic acid	29	92.1	1.8
17	Syringic acid	12	91.8	1.9
18	Epicatechin	29	85.3	2.4
19	Vanillin	1	86.8	2.8
20	p-coumaric acid	16	92.1	2.2
21	Ferulic acid	12	91.4	2.9
22	Rutin	3	85.0	1.6
23	o-coumaric acid	2	94.5	1.7
24	Myricetin	0.5	9.5	50.8
25	Resveratrol	1	75.9	3.8
26	Quercetin	1	34.4	14.0
27	Kaempferol	1	80.7	3.4

Table 4.1: Composition of the artificial wine sample. Recoveries and repeatability's (n=6) of the SPE method on Strata SDB-L (see Results and Discussion).

4.3 Results and Discussion

This study is aimed at obtaining chemical fingerprints of the non-volatile fractions of South African wines. As a first approach, direct injection of wine samples was evaluated, since, if feasible, this would provide the simplest method to screen a large amount of samples. It was found, especially in the case of red wines, that direct injection yielded complicated chromatograms in which interference from other wine constituents placed doubt on integration data. For the analysis of organic acids and sugars, the phenolic compounds were primarily responsible for interferences, since the modes of detection are not selective in these cases. On the other hand, the chromatogram for the phenolic compounds was found to be too complex because of the presence of anthocyanins and high molecular weight polyphenols (the tannins). Currently the wine tannins cannot directly be separated by any chromatographic technique. In reversed phase LC they elute as a broad bump, complicating the quantitation of the monomeric phenolics. In addition, the monomeric anthocyanins elute as very broad peaks, since the conversion between the different forms of these compounds at the pH of the mobile phase is very slow. The aim of this study was to develop a simple and reliable SPE method to be used for simultaneous clean-up of wine samples for the analysis of organic acids, sugars and monomeric polyphenols. For the latter, the specific aim was to be able to quantify as many compounds as possible in a single analysis, while excluding interference from anthocyanins and the tannins.

The generic SPE procedure applied to wine analysis is based on the retention of the hydrophobic constituents on reversed phase material, while the polar compounds are eluted with aqueous solutions. Although anion exchange cartridges have been used to separate neutral from acidic polyphenols [30, 31], by far the most commonly used sorbent is C_{18} [27-29, 33, 36]. In addition, polymer based sorbents have been applied to enrich polyphenols in sherry wine [37] and resveratrol in red wine [38]. Polymer based material has the advantage of being stable in the pH range 1-13, which is especially useful since the pH of eluents can be varied to achieve selective elution/retention. Increased retention of phenolics on polystyrene-

divinylbenzene (SDB) cartridges has been reported by Chilla et *al.* [37]. Recoveries were high but only measured for phenolic acids. Also, the reproducibility of the method, even with automation, was quite poor. In initial experiments tC_{18} and Strata SDB-L cartridges have been compared. The cartridges were preconditioned with 3 mL methanol and 3 mL water (pH 2.5). 5 mL of a wine sample previously adjusted to pH 2.5 was loaded, before rinsing with 2 mL water (pH 2.5). Each cartridge was then eluted with 5 mL ethyl acetate. These fractions were evaporated and redissolved in 2 mL 50% acetonitrile/50% water for injection. The cartridges were then eluted with 2 mL methanol to determine whether some solutes remained on the cartridge. The chromatograms of the ethyl acetate extracts are compared in figure 4.1.

It is evident that the recovery of the more polar compounds (the earlier eluting peaks) is much better on the SDB cartridge. The methanol fraction in both cases contained only the high molecular weight phenolics, eluting as a broad hump. Based on these results, consequent optimisation of the method was performed on SDB material.

During optimisation it was also found that if 1 mL sample is loaded, less breakthrough of phenolic compounds occurs resulting in less interfering peaks in the chromatograms for the acids and the sugars. Since the sensitivity of each separation procedure was sufficient for wine analysis, dilution of samples was not a problem. For the elution step, 4 × 1 mL of 20 mM sulphuric acid was found to be optimal, since this gave good recoveries for the organic acids, while the phenolic acids remained sufficiently retained. Ethyl acetate has been shown to be an effective solvent for elution of low molecular weight phenolics from reversed phase SPE materials, while simultaneously ensuring retention of the polymeric phenolics [28, 33]. A volume of 10 mL proved to be optimal, ensuring high recoveries for most of the phenolic standards. Calculated recoveries of 27 standard compounds, and the repeatability's of the method are summarised in table 4.1.

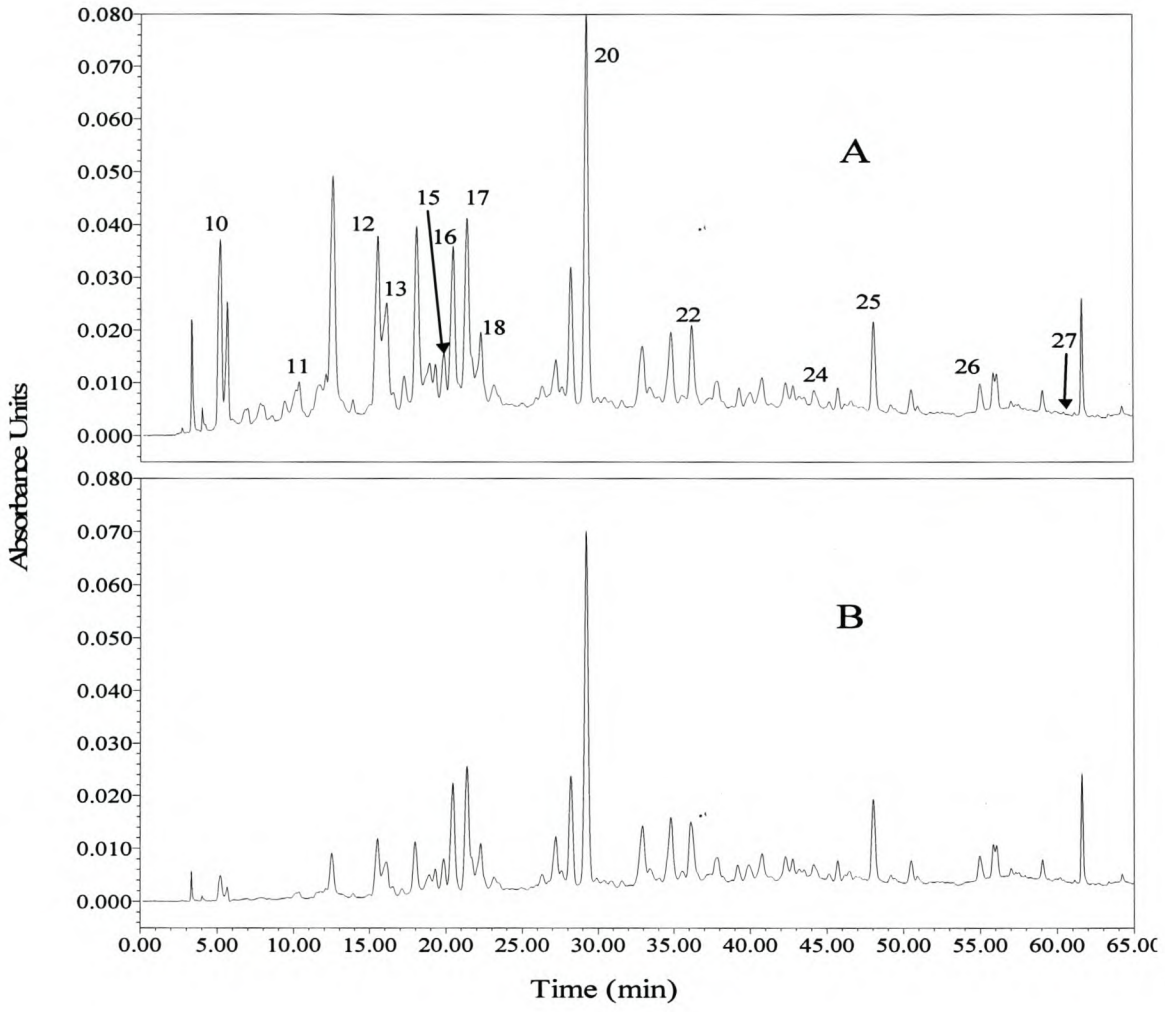

Figure 4.1: Comparison of enrichment of polyphenols on (A) styrene-divinylbenzene and (B) C_{18} SPE cartridges. Detection wavelength at 280 nm. Peak identification see table 4.1.

For the organic acids and sugars good recoveries with excellent repeatability's are obtained. For the phenolic compounds, acceptable recoveries over the whole range of compounds are achieved with exception of gallic acid, and the flavonols myricetin and quercetin. The lower recovery for gallic acid is still sufficient to quantify this solute in wine samples, as the

repeatability is good and gallic acid is present in all wine samples at high concentration. The reason for the poor recoveries and repeatability's of some of the flavonols is unclear at this stage. However, in comparison with methods reported in the literature, the proposed SPE procedure offers better recoveries and repeatability's over the whole range of compounds analysed. In addition, samples suitable for the reliable analysis of three different classes of essential wine compounds are produced using a single SPE procedure. The procedure optimised on Strata SDB-L was repeated on Chromabond HR-P, both SDB materials. It was found that recoveries for some phenolics and most organic acids were much lower on Chromabond HR-P compared to Strata SDB-L. In other words, the method presented here, can be used reliably only on Strata SDB-L cartridges, since it seems that there is variability between cartridges from different manufacturers.

A comparison between direct injection of a 4 times diluted wine sample and a SPE sample for organic acid analysis is presented in figure 4.2. It is clear that interference free analysis is possible after using the proposed SPE method, whereas quantitation of certain compounds is problematic in the case of direct injection.

Similarly, direct injection and the proposed SPE procedure for the analysis of monomeric polyphenols are compared in figure 4.3. SPE is capable of reducing interference from the high molecular weight phenolics eluting as a broad hump, while at the same time retaining most of the information on the low molecular weight compounds. Figure 4.4 presents the analysis of fructose and glucose in a dry white wine by NP-LC-ELSD after SPE clean-up.

Figures of merit for each LC method are summarised in table 4.2. The proposed procedures are presently applied for the characterisation of South African wine samples by principal component analysis (Chapter 11).

No.	Compound	Linear range[a]	m / a; b[b]	R^2	%RSD (m)[c]	LOD[d]	LOQ[e]
1	Citric acid	100-5000	0.001346	0.9998	1.1	1.5	5.1
2	Tartaric acid	100-5000	0.001196	0.9998	1.0	1.1	3.7
3	Malic acid	100-5000	0.000836	0.9996	1.7	2.2	7.5
4	Succinic acid	100-5000	0.000689	0.9997	0.8	3.7	12.5
5	Lactic acid	100-5000	0.000685	0.9996	1.7	3.7	12.2
7	Acetic acid	100-5000	0.000593	0.9997	1.0	4.6	15.5
8	Fructose	100-5000	2708; 1.311	0.9925	-	19	62
9	Glucose	100-5000	1730; 1.402	0.9929	-	22	73
10	Gallic acid	0.5-50	57702	0.9999	0.4	0.03	0.09
11	Protocatechuic acid	0.5-50	26929	1.0000	0.3	0.07	0.23
12	Catechol	0.5-50	21108	1.0000	0.3	0.08	0.26
13	Catechin	0.5-50	12421	0.9999	1.6	0.15	0.51
14	Chlorogenic acid	0.5-50	54773	0.9999	0.9	0.04	0.15
15	Vanillic acid	0.5-50	31544	1.0000	0.4	0.07	0.23
16	Caffeic acid	0.5-50	104124	1.0000	0.6	0.02	0.06
17	Syringic acid	0.5-50	56343	1.0000	0.5	0.04	0.13
18	Epicatechin	0.5-50	13718	0.9999	0.6	0.15	0.50
19	Vanillin	0.5-50	78629	1.0000	0.5	0.03	0.11
20	p-coumaric acid	0.5-50	125562	1.0000	0.5	0.02	0.05
21	Ferulic acid	0.5-50	105796	1.0000	0.5	0.02	0.06
22	Rutin	0.5-50	24117	1.0000	0.6	0.18	0.60
23	o-coumaric acid	0.5-50	110773	1.0000	0.4	0.02	0.08
24	Myricetin	0.5-50	61389	0.9995	5.4	0.15	0.51
25	Resveratrol	0.5-50	152755	1.0000	0.5	0.01	0.05
26	Quercetin	0.5-50	69745	0.9997	0.9	0.09	0.31
27	Kaempferol	0.5-50	74429	1.0000	1.1	0.05	0.17

[a] ppm

[b] m = slope of a linear calibration curve; a, b are coefficents for exponential ($y = ax^b$) calibration curves

[c] relative standard deviation of the slope, determined over a period of 4 months (n = 4)

[d] Limit of detection in ppm, determined at a S/N level of 3

[e] Limit of quantitation in ppm, determined at a S/N level of 10

Table 4.2: Summary of linearity and sensitivity of each LC method.

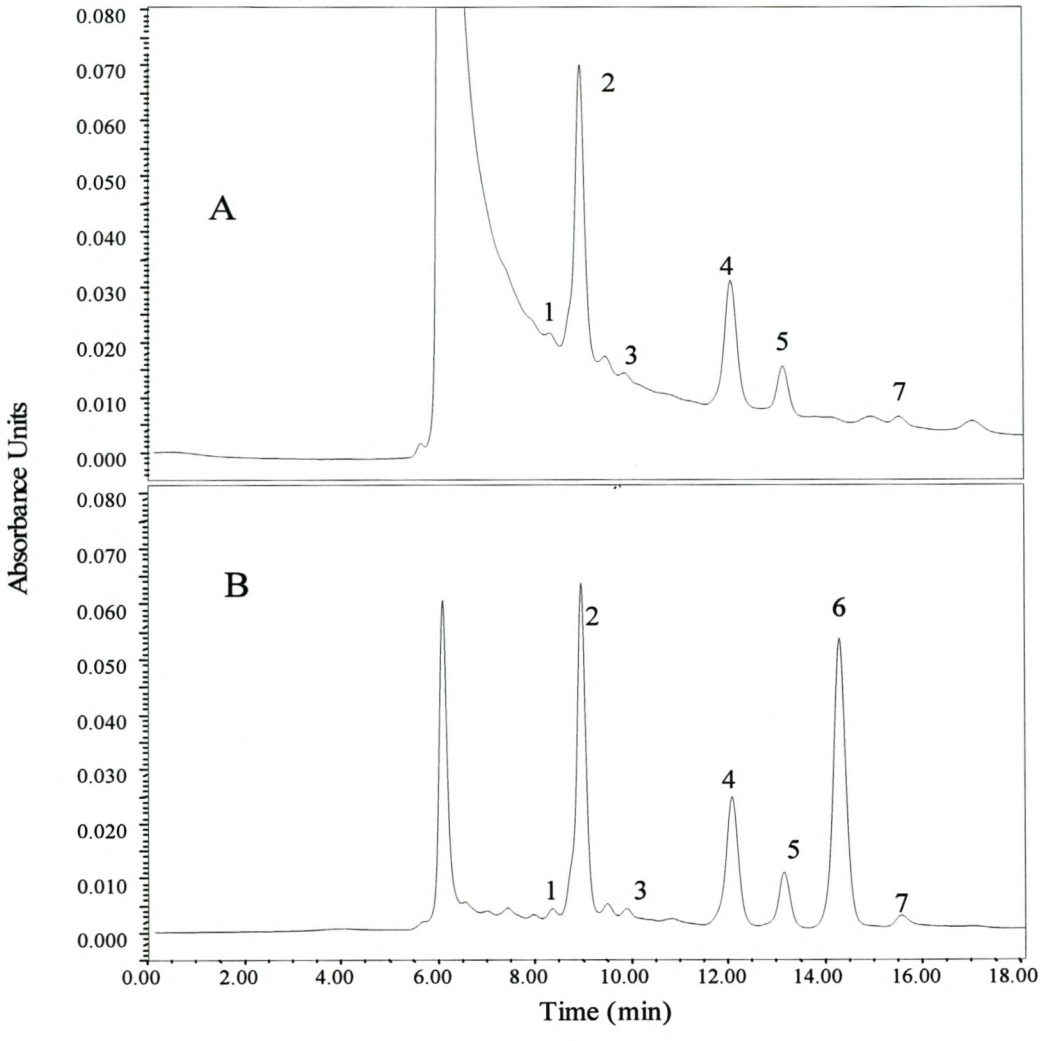

Figure 4.2: Analysis of organic acids in South African red wine. (A) by direct injection (diluted 1:4), and (B) after SPE clean-up. Peak identification see table 4.1.

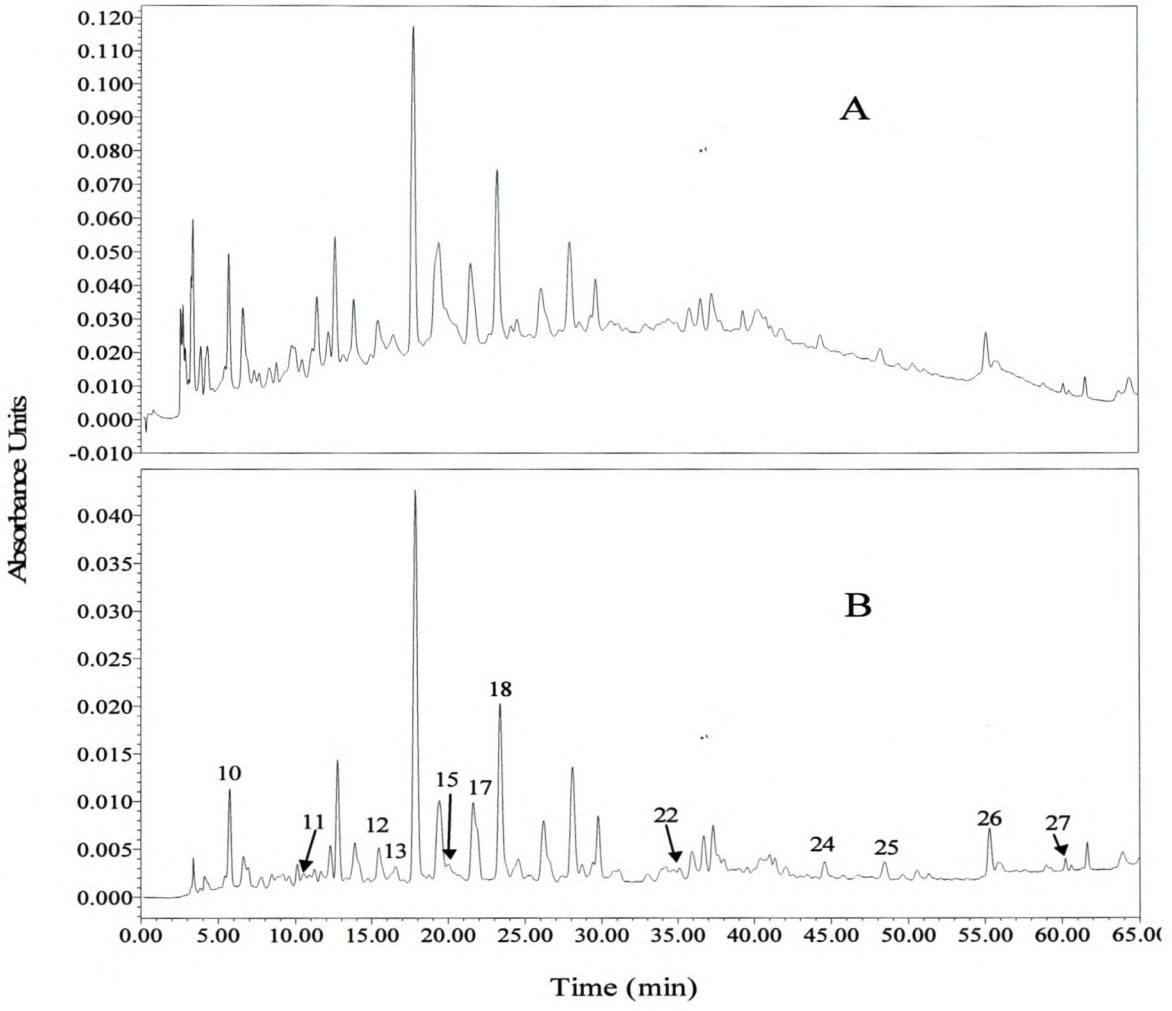

Figure 4.3: Analysis of polyphenols in South African red wine. (A) by direct injection, and (B) after SPE clean-up. Peak identification see table 4.1.

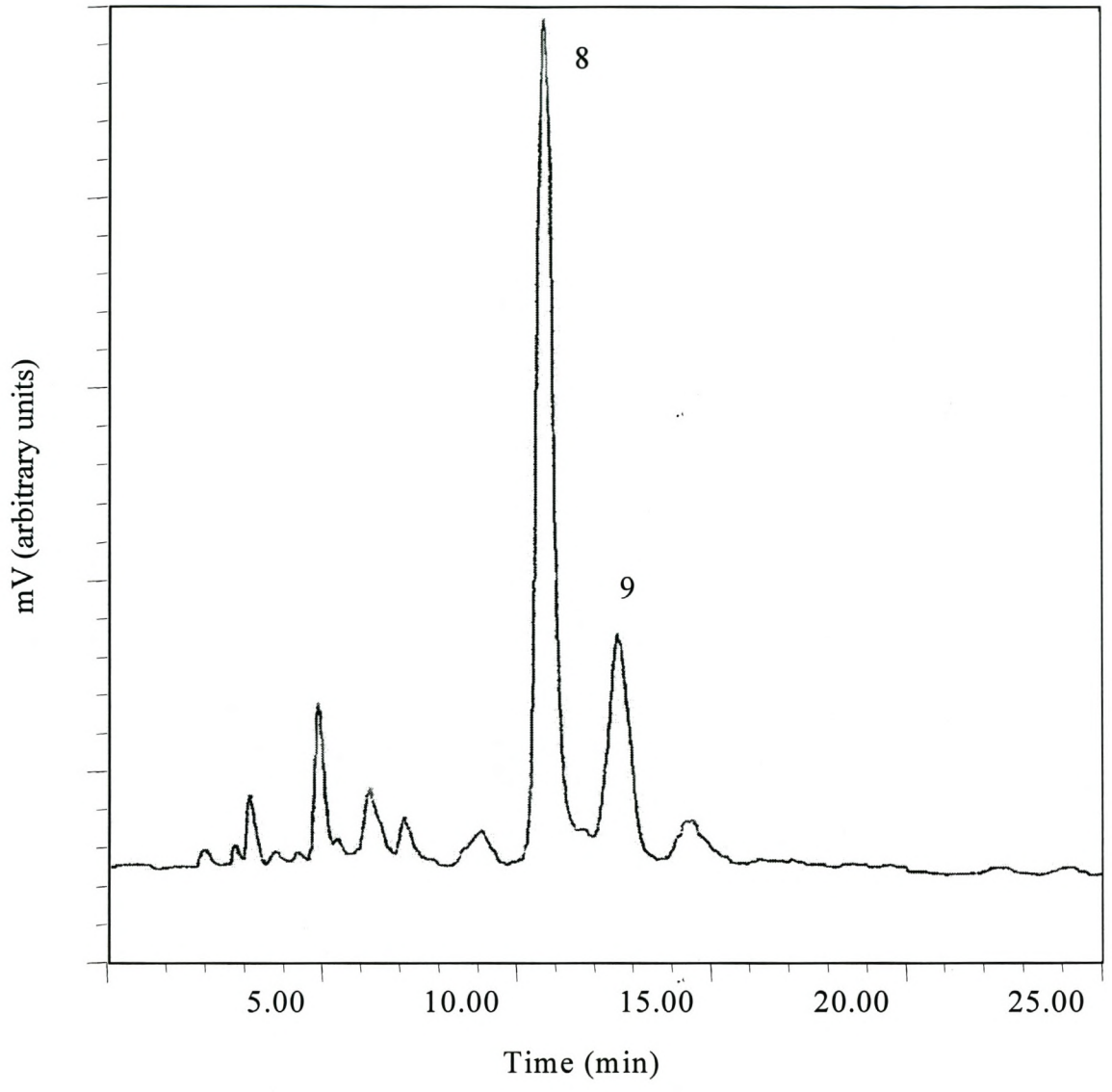

Figure 4.4: Analysis of sugars in South African dry white wine after SPE clean-up. Peak identification see table 4.1.

4.4 Conclusions

A SPE procedure for fractionation/clean-up of wine samples, suitable for the analysis of organic acids and sugars, as well as monomeric polyphenols has been developed. Styrene-divinylbenzene cartridges provided better retention of the phenolics than the more commonly used C_{18} cartridges. Due to the high recoveries over the whole range of analysed compounds, the low %RSD's, and the fact that monomeric phenolics can be analysed in a single run, the method represents an improvement on currently used SPE procedures, and, moreover is suitable for routine wine analysis.

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5

Capillary Electrophoresis Method for the Determination of Organic Acids in Wine*

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5.1 Introduction

Organic acids play a major role in determining the sensory properties and microbiological as well as physicochemical stability of wines [1]. Knowledge of the specific organic acid content of grapes, musts and wines is important to the winemaker as the data provide valuable information on the optimum harvest period, the progress of fermentation and the overall quality of the wine. For this reason a rapid and reliable analytical method for the determination of these acids is of great importance to the wine industry.

Chromatographic techniques represent a promising alternative to the more laborious enzymatic methods for these analyses. In this regard the use of various modes of Liquid Chromatography (LC) for these analyses has been reported. These include reversed phase LC with UV detection [2-8]. However, these methods suffer from poor sensitivity and selectivity, and in most cases derivitization is required to improve the results, thereby increasing the total analysis time. Better separation is achieved using ion-exclusion chromatography [9-12], where the analytes are separated by a combination of ion-exclusion and partitioning processes. UV and/or Refractive Index (RI) are the detection methods of choice in this case. This technique couples the advantages of ruggedness and sufficient selectivity for the determination of organic acids in a variety of natural products. However, expensive columns and harsh operating conditions do detract from the overall usefulness of the method. Moreover, in the case of complex samples such as red wines, sample clean-up is often required for reliable quantitation.

Capillary Electrophoresis (CE) has increasingly been shown to be an attractive alternative to LC for the analysis of natural products [13, 14]. Advantages of the former method include increased speed and efficiency of separation, low column costs and sample/solvent consumption. Specifically the analysis of organic acids by capillary zone electrophoresis (CZE) with indirect UV detection has received a lot of attention [15-22]. The choice of background electrolyte (BGE) is of crucial importance, and several alternatives have been proposed [14, 18]. Soga and Ross introduced 2,6-pyridinedicarboxylic acid (PDC) as BGE for the determination of organic and inorganic anions, amino acids and carbohydrates in a variety

of samples [19, 20]. The method has the advantage of being fast, sensitive, reliable and quantitative, while simultaneously masking the effect of metals in the system that might otherwise interfere with the analysis of some organic acids. Kandl and Kupina applied this method for the analysis of wine and grape juice [21]. The method allowed accurate determination of organic acids in these samples with only dilution required prior to injection. However, severe restrictions regarding the linearity of acetic and succinic acids were reported. The phenomenon responsible for the non-linearity has been elucidated and an improved CZE method employing a PDC BGE for the determination of organic acids in wines is presented.

5.2 Experimental

5.2.1 Materials

L-malic acid, tartaric acid, ethelenediaminetetracarboxylic acid (EDTA) and sodium azide were purchased from Sigma-Aldrich (Atlasville, South Africa). Acetic acid and lactic acid were from Riedel-de Haën (Midrand, South Africa), succinic acid (disodium salt), formic acid and PDC from Acros (Geel, Belgium) and citric acid from Merck (Darmstadt, Germany). 20 mM sulphuric acid (Merck) used as LC mobile phase was filtered through 0.45 μm HV filters before use (Millipore Corporation, Bedford, MA). Buffers for CE were filtered through disposable syringe filters (Millex HN 0.45μm Nylon, Millipore). The SPE cartridges (Strata SDB-L styrene-divinylbenzene, 3 mL, 500 mg phase) were from Phenomenex (Torrance, CA, USA). Wine samples were purchased from local stores. If not analysed directly, the samples were transferred under nitrogen to completely filled amber bottles.

5.2.2 Instrumentation

CE and data analyses were performed on a HP^{3D} CE capillary electrophoresis system equipped with diode array detection from Agilent (Waldbronn, Germany). Bare fused silica capillaries

(Composite Metal Services Ltd., Worcester, UK) with an internal diameter of 75 μm and a total length of 80.5 or 111.3 cm were used. In the final conditions a 7.5 mM PDC buffer containing 0.5 mM CTAB and 0.5 mM EDTA was prepared by dissolving the appropriate amounts of chemicals and adjusting the pH to 5.60 with 1 M NaOH. Injection was performed either in the pressure mode by applying 50 mbar pressure for 3 s or in the electrokinetic mode by applying –10kV for 2 s. In both cases a buffer plug was injected (50 mbar, 2 s) after the sample. Between runs the capillary was flushed for 4 minutes with buffer, followed by a voltage conditioning step (-15 kV for 1 minute). The separation voltage was ramped from 0 to –22 kV in 0.5 minutes. Indirect UV detection was performed at 350 nm with a reference wavelength of 210 nm. The capillary temperature was set to 15 °C.

LC analyses were carried out on an Alliance 2690 Separations Module equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). Data analysis was done using Millenium[32] Chromatography Manager software (Waters). The column (Aminex HPX-87H Ion Exclusion Column, 300 mm x 7.8 mm) and guard column of the same phase were a kind gift from Bio-Rad (Nazareth, Belgium). The mobile phase consisted of an aqueous solution of 20 mM H_2SO_4. All experiments were performed at a flow rate of 0.6 $mL \cdot min^{-1}$. The injection volume was 10 μL and the column temperature was kept constant at 50 °C. Detection was performed at 210 nm.

5.2.3 Preparation of Standard Solutions and Samples for CE

Formic acid was used as internal standard for both CE and LC experiments. Calibration samples of 5000, 2500, 1000, 500 and 100 ppm of all 6 acids were prepared in deionised water. These were diluted 80 × with a 25.32 ppm formic acid solution to produce samples containing 25 ppm of the internal standard. Wine samples were diluted in the same manner.

5.2.4 Preparation of Standard Solutions and Samples for LC

The same calibration levels were used in LC, without dilution. Each sample contained 1000 ppm formic acid as internal standard. The wine samples were purified by SPE. The styrene-divinylbenzene cartridges were conditioned with 3 mL each of ethyl acetate, methanol and water (pH 2.5). The pH of each wine sample was adjusted to 2.5 with 6 M HCl prior to spiking with 5000 ppm formic acid. 1 mL sample was loaded onto the cartridges. The organic acids were rinsed of with 4 x 1 mL of 20 mM sulphuric acid, and the combined (5 mL) eluent was injected. Recoveries determined using an artificial wine sample were used for quantitative calculations.

5.3 Results and Discussion

5.3.1 Choice of Operating Conditions

A BGE consisting of PDC at pH 5.60 and containing 0.5 mM CTAB provides sufficient sensitivity and resolution for the analysis of the 6 major wine acids [21]. When the optimum buffer concentration is chosen the following aspects have to be considered: the concentration of the buffer (and visualising ion) should be kept at a minimum in order to reduce baseline noise, while at the same time being sufficient to ensure effective separation, sensitivity and buffering capacity. Kandl et *al.* considered 5 mM PDC to be the minimum concentration needed for reproducible migration times. In this study, formic acid was used as internal standard in both the CE and LC analyses. It was found that a PDC concentration of 7.5 mM was needed to provide baseline separation of formic acid from tartaric acid. For this BGE the best signal-to-noise ratios for the acids were obtained by using a signal wavelength of 350 nm with a reference wavelength of 210 nm. A 75 μm i. d. capillary of length 111.3 cm and a working temperature of 15 °C were chosen in order to achieve acceptable sensitivity while limiting the generated current (and concurrent Joule heating). Although the 6 wine acids could be separated under these conditions, two problems were encountered: the citric acid peak

displayed severe tailing, increasing the limit of detection and complicating quantitation, and, as reported by Kandl et *al.*, detector response for acetic and succinic acids was only linear up to 750 and 1500 ppm, respectively.

5.3.2 Determination of Citric Acid

It has been reported that the presence of metal cations in the separation system does affect the migration times and peak areas of certain acids. For some BGEs the acids with high stability constants may even be lost at lower concentrations [19, 22]. In the case of wine acids, this will predominantly affect the analysis of citric acid because of the higher stability constant of metal complexes formed with this acid. However, Soga and Ross reported that the complexing ability of PDC is sufficient to mask the effect of trace metals present in the capillary by preferential complexation [19]. Moreover, Kandl et *al.* reported no such problems when they applied this method to wine analysis [21]. During initial experiments in this study badly tailing peak shapes for citric acid were observed. Increasing the PDC concentration up to 20 mM did not improve the situation. In order to evaluate whether this was the result of metals present in the system, EDTA was added to the BGE. Because of the high stability constant of EDTA-metal complexes, this should remove any interferences from metals. Indeed, peak height for citric acid increased with the EDTA concentration from 0.05 to 0.5 mM, and 0.5 mM was consequently chosen as the optimum concentration. Contrary to previous reports, the complexing ability of PDC was insufficient (up to the 20 mM level) to eliminate interference from metal cations in our study. The effect of the addition of EDTA on the peak shape of citric acid is demonstrated in figure 5.1.

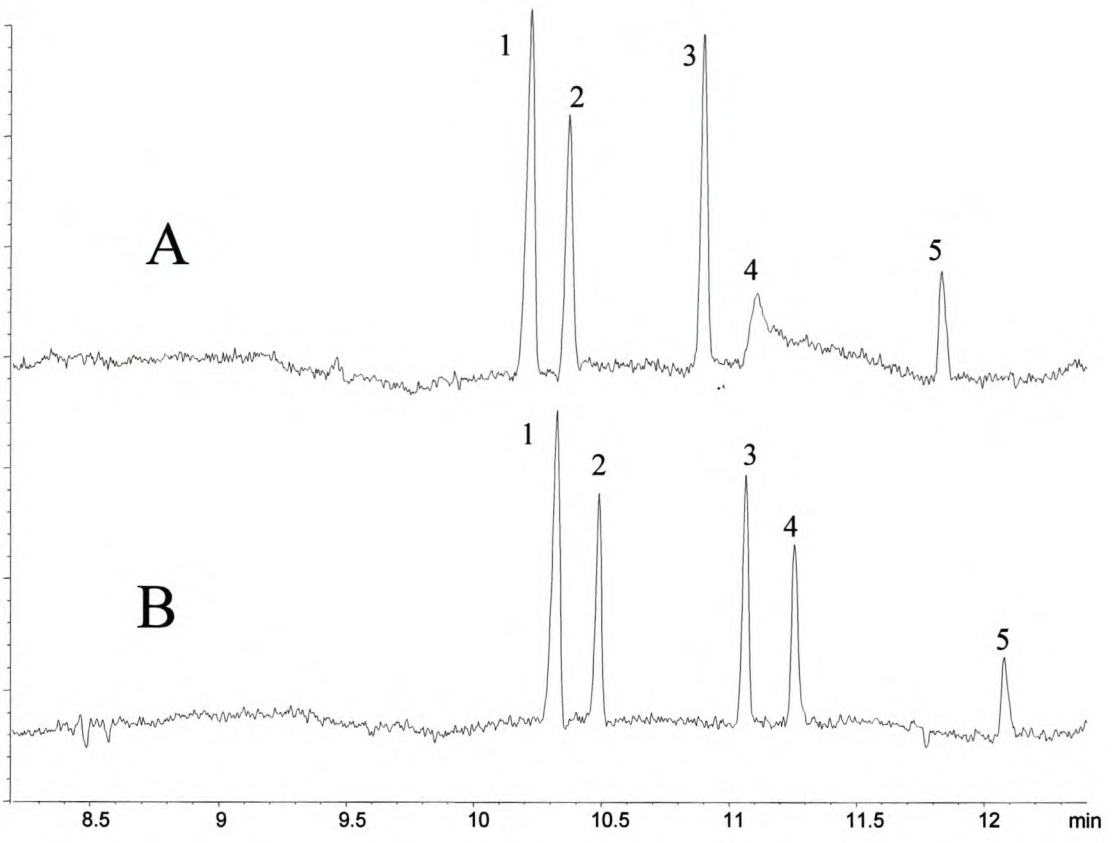

Figure 5.1: Effect of EDTA on the peak shape of citric acid: (A) 7.5 mM PDC, 0.5 mM CTAB, pH 5.6, (B) 7.5 mM PDC, 0.5 mM CTAB, 0.5 mM EDTA, pH 5.6. Sample: 1000 ppm organic acid standards, diluted 40 ×. Capillary: 75 μm i.d., 80.5 cm L_{tot}. Injection: 50 mbar for 3 s. Other conditions as described in text. Peaks: 1 = formic acid (I.S.), 2 = tartaric acid, 3 = malic acid, 4 = citric acid, 5 =succinic acid.

5.3.3 Improving the Linearity of the Method

Kandl and Kupina suggested that the lower linear range for acetic and succinic acid was the result of a "saturation effect to effectively displace the visualising ion". We observed similar problems during initial experiments employing pressure injection. It was noted that the peaks for these two acids became severely distorted to the point of being split at the higher calibration levels. In addition, when the calibration graphs for these acids were constructed individually (without the addition of the other 5 acids), the responses were linear over the

whole range. Initially we thought this was a result of mismatch in conductivities between the sample plug and the BGE, with the former being significantly higher at the higher calibration levels. This could cause a form of de-stacking, where those solutes in the buffer accelerate away from the sample plug (this process is the opposite to that occuring during field-amplified sample stacking), leading to the observed split peaks. However, this would not explain why the effect is only significant for acetic and succinic acids. In other words, the difference in mobility in the sample and buffer regions is greater for these two acids than for the others. This can be explained by looking at the pH of the sample and the pKa's of the acids (table 5.1). Since the pH of the sample is lower than that of the buffer, most acids will have higher charge-to-mass ratios in the buffer region. Acetic and succinic acid have the highest pKa_1 values of all the acids. Accordingly, only a small percentage of these acids are charged in the sample region, whereas both possess at least a -1 charge in the buffer. This translates into large difference in the electrophoretic mobilities (μ_e) of these acids between the two regions, and can explain why split peaks are observed for these acids (those solutes in the buffer region accelerate away from the sample zone). The rest of the acids are all ionised to greater extent in the sample zone, and thus the difference in mobilities between the two phases is not as pronounced. The effect will become more pronounced as the pH of the sample drops with increasing acid content, which explains why the problem does not occur at lower calibration levels or when these acids are calibrated individually.

	pKa_1	pKa_2	pKa_3	Calibration level	Sample pH	
formic	3.74			5000	3.39[a]	3.49[b]
tartaric	3.04	4.37		2500	3.47[a]	3.59[b]
malic	3.46	5.10		1000	3.51[a]	3.70[b]
citric	3.13	4.76	6.40	500	3.55[a]	3.80[b]
succinic	4.21	5.64		100	3.70[a]	4.03[b]
acetic	4.76					
lactic	3.86					

[a]After 40x dilution with the internal standard solution
[b]After 80x dilution with the internal standard solution

Table 5.1: pKa's of the six major wine acids and pH's of calibration samples after dilution.

Figure 5.2 demonstrates the effect of sample pH on the peak shape of succinic and acetic acids: a 5000 ppm calibration sample was diluted 40 × and injected in the pressure mode (A) with the pH unadjusted at 3.39 and (B) with the pH adjusted to 5.6. The disappearance of split peaks for both acids is evident.

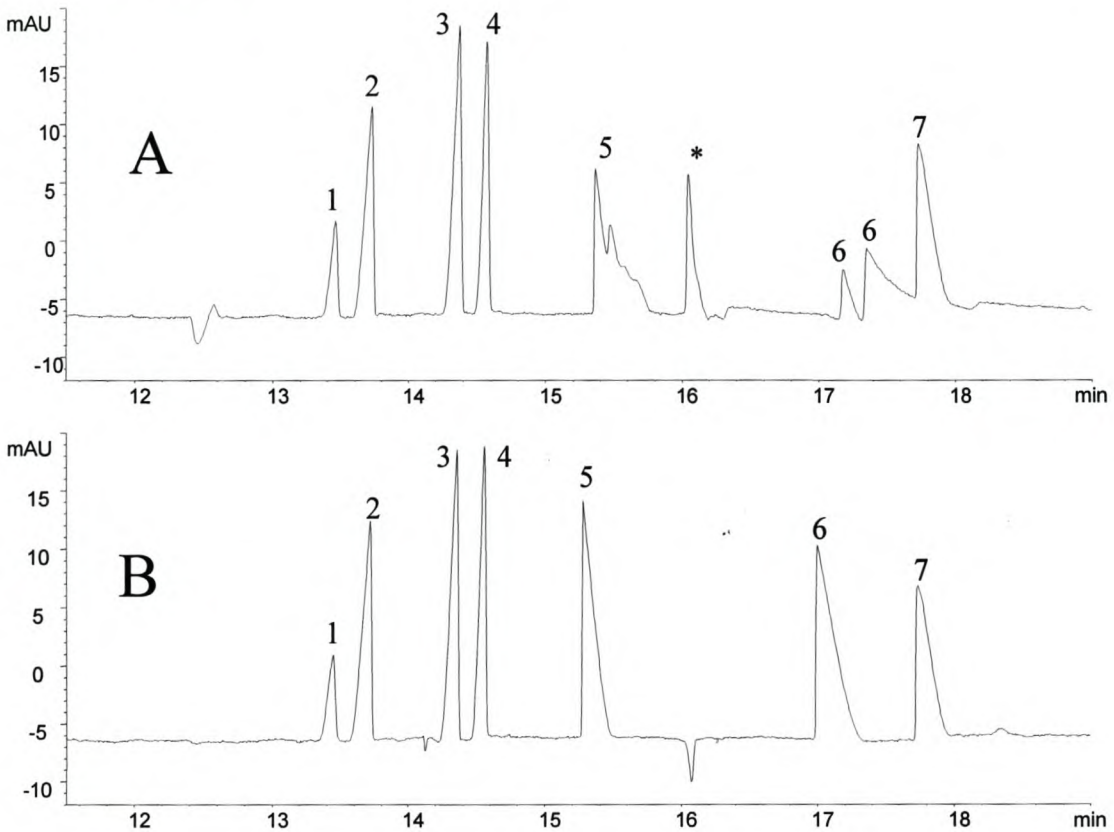

Figure 5.2: Effect of sample pH on the peak shape of acetic and succinic acids. Sample: 5000 ppm organic acid standards, 40 × diluted (A) pH unadjusted at 3.39, (B) pH adjusted to 5.6. BGE: 7.5 mM PDC, 0.5 mM CTAB, 0.5 mM EDTA, pH 5.6. Capillary: 75 μm i.d., 111.3 cm L_{tot}. Other conditions as in figure 5.1. Peaks: 1 = formic acid (I.S.), 2 = tartaric acid, 3 = malic acid, 4 = citric acid, 5 =succinic acid. 6 = acetic acid, 7 = lactic acid, *EDTA system peak.

To our knowledge, this is the first time that the effect of sample pH on the de-stacking of solutes has been reported. The phenomenon also suggests the opportunity for stacking solutes

by increasing the pH of the sample, in cases where the solutes are only partially charged in the BGE.

As an alternative to adjusting the sample pH, we investigated the use of electrokinetic injection. During this mode of injection the solutes are loaded onto the capillary by a combination of their electrophoretic mobilities and the electro-osmotic flow (EOF). Under the operating conditions of the method, i.e. reversed EOF, the electrophoretic mobilities of the organic acids are in the same direction as the EOF. It was found that the amount of organic acids loaded onto the capillary when employing –10 kV for 2s, is significantly higher than for pressure injection. Also, the responses for all the acids were linear up to 2500 ppm. When using an 80 × instead of a 40 × dilution, linear responses for all 6 acids over the whole calibration range were obtained, while simultaneously improving the limits of detection (LOD). Peak shapes for acetic and succinic acids were normal over the whole range. The problems encountered with pressure injection are most likely overcome by a combination of 2 factors: the further dilution increases the sample pH, while the lower applied voltage during electrokinetic injection reduces the difference in the velocity of acetic and succinic acid between the sample and buffer zones (since $v = \mu_e E$). The reproducibility of the finalised method was investigated by injecting 7 dilutions of the same wine. These results are summarised in table 5.2.

	Slope	Linear Range[a]	r^2	LOD (pressure)[b]	LOD (electro-kinetic)[a]	%RSD (migration time)[c]	%RSD (corrected peak area)[c]
formic						0.13	1.9
tartaric	0.000266	100-5000	0.9984	50.8	25.0	0.14	1.2
malic	0.000233	100-5000	0.9985	46.9	30.6	0.14	1.5
citric	0.000181	100-5000	0.9961	56.6	75.0	0.15	5.0
succinnic	0.000162	100-5000	0.9985	123.5	37.8	0.15	5.0
acetic	0.000117	100-5000	0.9952	71.4	46.4	0.12	4.9
lactic	0.000152	100-5000	0.9956	28.6	28.5	0.08	5.1

[a]Amounts in ppm, prior to 80x dilution and electrokinetic injection
[b]Amounts in ppm, prior to 40x dilution and pressure injection
[c]Relative standard deviation, n = 7

Table 5.2: Analytical performance of the proposed method.

5.3.4 Analysis of South African Wines

The optimised CE method was used to analyse 20 South African red and white wines. The results were compared to those obtained for the same wines using ion-exclusion LC. Examples of an electropherogram and chromatogram for a red wine are depicted in figures 5.3 and 5.4, respectively. From these figures it is clear that the baseline separation of all acids obtained by CE cannot be matched by the LC method, even though the sensitivity of the latter is better.

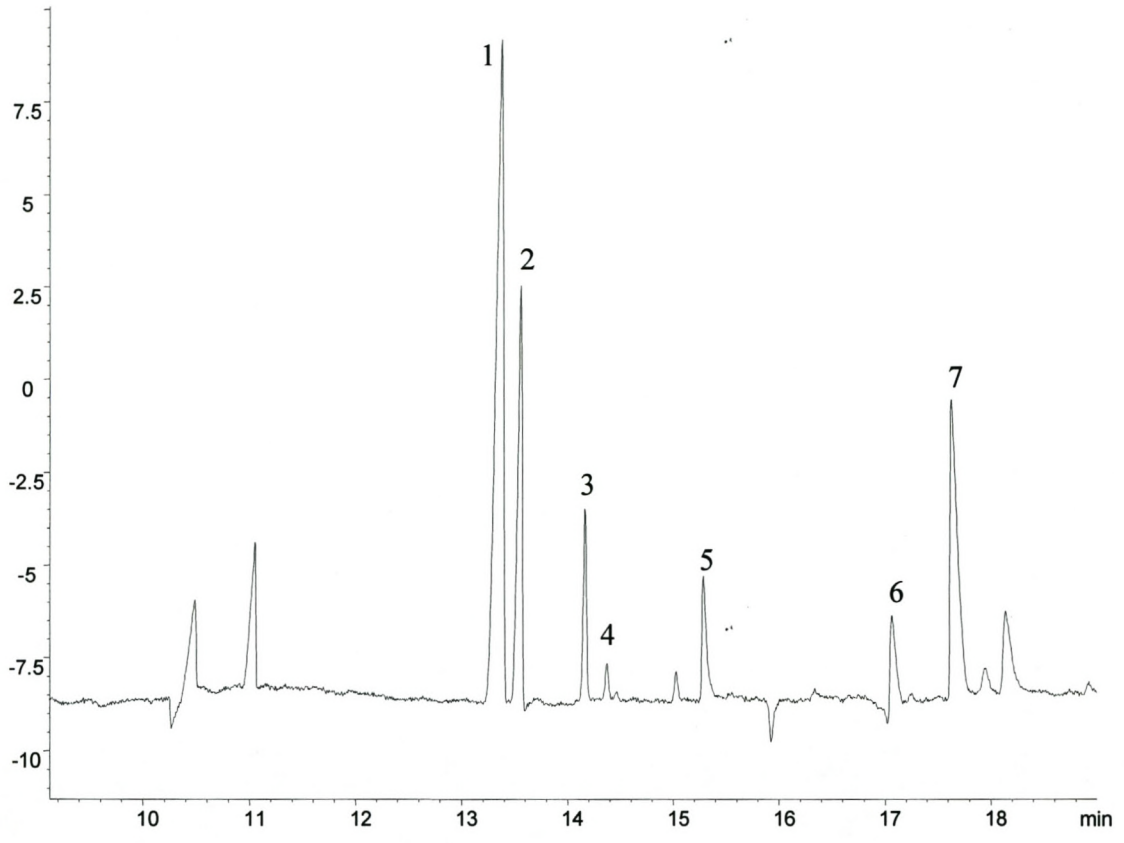

Figure 5.3: Optimised CE separation of South African wine (Red Blend 2002b, diluted 80 ×). Injection: -10 kV, 2s. Other conditions as in figure 5.2.

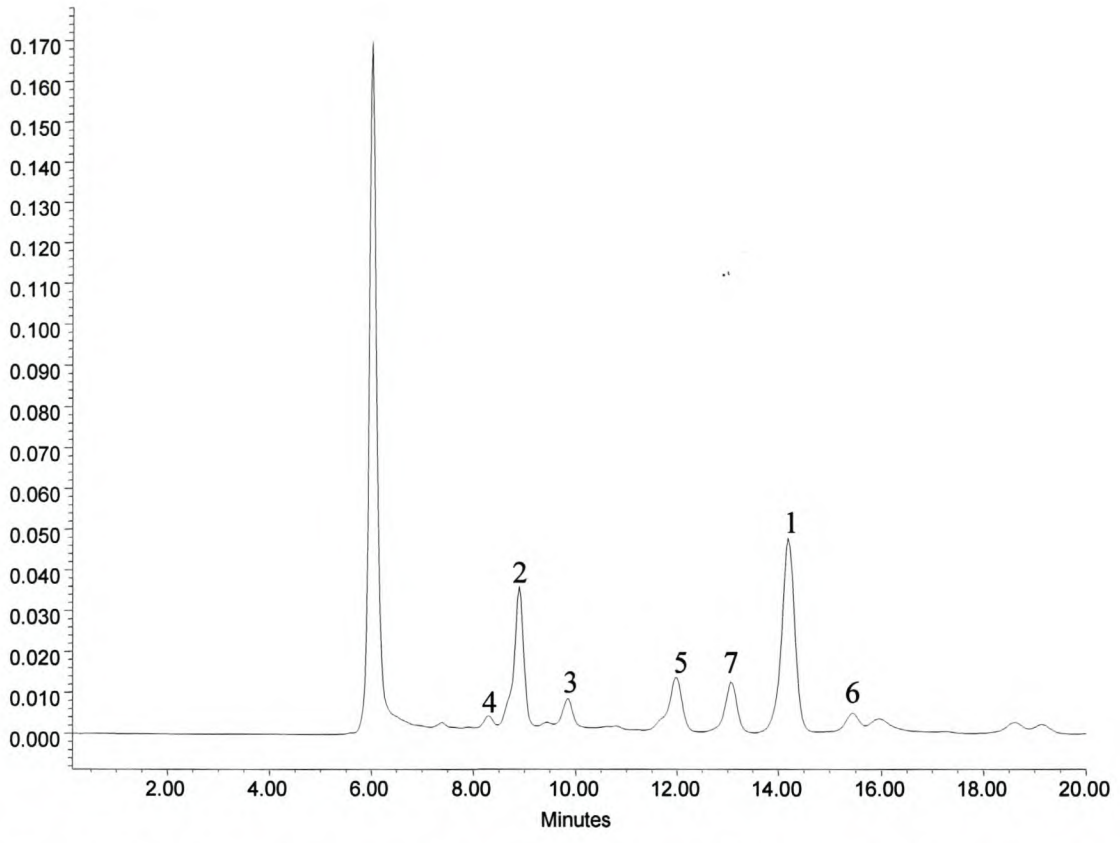

Figure 5.4: Ion-exclusion LC analysis of Red Blend 2002b, after SPE clean-up. Conditions as specified in text. Peak numbering as in figure 5.2.

Quantitative results using both methods are summarised in table 5.3. It is evident that quantitative results between the 2 methods are in good agreement, with a few exceptions. For tartaric acid, excellent agreement between both methods is achieved. No over-estimation of tartaric acid by CE was observed for these wines, as was reported by Kandl and Kupina because of co-elution of fumaric acid. For malic and citric acid, good agreement at higher concentrations was achieved. For wines containing these acids in small amounts, the values obtained by LC are generally higher, mainly due to difficult integration of the small peaks in the chromatogram. Succinic acid was constantly overestimated by the LC method by a factor of 3-10. From the chromatogram and the UV spectrum of the peak it was clear that this is the result of another compound co-eluting with succinic acid. Acetic and lactic acids values were

consistently slightly higher by the CE method. In conclusion, the CE method was found to be more reliable, with the added advantage of not requiring sample clean-up.

Wines	Tartaric		Malic		Citric		Succinic		Acetic		Lactic	
	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE
Cabernet Sauvignon 1988	1302	1297	40	72	44	n.d.	8694	958	877	953	2388	2476
Cabernet Sauvignon 1992	1294	1099	187	222	70	n.d.	10499	1443	658	756	2203	2398
Cabernet Sauvignon 1994	1371	1335	61	81	45	n.d.	7974	1231	630	798	1942	2299
Cabernet Sauvignon 1995	1151	1057	58	38	46	n.d.	9778	1411	645	835	2400	2596
Cabernet Sauvignon 1996	1502	1559	58	31	40	n.d.	7634	1145	837	1069	3758	3959
Cabernet Sauvignon 1997	1730	1771	77	44	41	n.d.	9379	1588	736	922	2918	3154
Cabernet Sauvignon 1998	1366	1411	44	41	42	n.d.	8805	1240	948	1081	2825	3204
Cabernet Sauvignon 1998b	1495	1546	68	34	34	n.d.	6893	1129	779	881	3383	3738
Red Blend 1997	1088	1099	70	59	50	n.d.	4799	1015	794	878	2811	2976
Red Blend 1998	1467	1269	45	39	24	n.d.	4928	1068	844	894	3732	4030
Red Blend 1999	1308	1360	105	82	61	n.d.	3554	997	832	949	3046	3295
Red Blend 2000	1372	1427	122	92	66	n.d.	4188	1001	818	977	2414	2722
Red Blend 2002	2566	2667	137	132	93	75	4161	998	328	465	1356	1762
Red Blend 2002b	1589	1560	690	585	177	136	2673	651	698	683	1793	2088
Merlot 1999	1884	1851	275	250	43	n.d.	4036	1286	676	532	1586	1862
Chardonnay 1999	1479	1610	656	636	29	n.d.	4400	510	580	597	2127	2865
Chardonnay 2000	1159	1209	1175	980	103	75	3714	505	497	515	1529	1882
Chardonnay 2001	964	923	2026	1700	84	n.d.	4342	477	332	414	1784	1762
Chardonnay 2002	1267	1280	2147	2170	249	233	2856	484	378	401	352	744
Sauvignon Blanc 2002	1874	1830	3103	3047	321	323	2460	407	619	651	158	612

Table 5.3: Comparison of quantitative data obtained by CE and LC for the organic acid analysis of 20 South African wines. All values are in $mg \cdot L^{-1}$.

5.4 Concluding Remarks

A reliable CE method for the determination of the 6 major organic acids in wines has been established. The method is based on the use of PDC as BGE. EDTA was added to improve the peak shape and quantitation of citric acid. Limitations regarding the linearity of the method reported by Kandl et *al.* were shown to be the result of a lower sample pH than that of the BGE. These problems were avoided while simultaneously increasing the LOD, by employing electrokinetic instead of pressure injection. 80 times dilution with an internal standard solution only was required before analysis. The method delivers sufficient sensitivity and reproducibility for wine analysis, and is considered more reliable than ion-exclusion LC.

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6

Comparison of Chromatographic and Electrophoretic Methods for the Determination of Carbohydrates in Wine

6.1 Introduction

Accurate determination of sugars is of great importance to the wine industry for a number of reasons. Glucose and fructose, the major hexoses present in grapes and must, are used to determine optimal grape ripeness. As primary substrates during alcoholic fermentation, these sugars are responsible for the formation of ethanol as well as a number of secondary products, and their concentrations are used to determine the endpoint of fermentation. Apart from their obvious contribution to the organoleptic profile of the wine, their concentrations can also vary during ageing and barrel storage, thus necessitating their determination throughout the winemaking process [1, 2].

While various analytical methods have been described even for routine carbohydrate analysis, each method has certain drawbacks, and most are suitable only for specific sample/analyte combinations. In the case of gas chromatography, where derivitisation is required, good efficiencies and sensitivity are obtained, but disadvantages include the formation of multiple, as well as unstable derivatives [3, 4]. While high performance liquid chromatography (HPLC) is nowadays routinely used for these analyses, drawbacks include inadequate resolving power in the case of complex samples, lack of sufficiently sensitive detectors, and often the need for extensive sample preparation. The various HPLC methods proposed can be divided into those employing (1) anion-exchange [2, 5-11], (2) cation-exchange [11-16] and (3) normal phase columns [10, 17-21]. Detection is performed using refractive index (RI) [6, 11-20] pulsed amperometric detection (PAD) [2, 8, 9] or evaporative light scattering detection (ELSD) [10, 21], or, in exceptional cases, absorbance-based detectors in combination with derivitisation reactions [5, 7]. In the case of wine samples, cation-exchange columns are commonly used together with a dilute acid mobile phase to simultaneously detect the organic acids and sugars. However, fructose and glucose are not well resolved from malic acid under these conditions, precluding their simultaneous determination in, for example, musts and sweet wines. The use of separate detection methods (RI for the sugars and UV detection for the acids) only slightly improves this situation. Another option is to separate the acids from the sugars off-line, using anion

exchange solid phase extraction (SPE) cartridges, although two analyses are needed for quantitation of both classes [14]. Similarly, SPE sample clean-up prior to anion-exchange-PAD analysis of especially red wines is required [8, 9]. For normal phase LC, direct injection or even dilution of wine samples have been used, although reliable quantitation is not guaranteed because of non-selective detection.

More recently a number of reports dealing with the application of capillary electrophoresis (CE) for sugar analysis have appeared [22, 23]. Principally two approaches to overcome the inherent problems of lack of charge and chromophore of neutral carbohydrates can be distinguished. The first involves derivitization to produce analytes possessing acidic or basic functional groups together with a chromophore suitable for UV or fluorescence detection [24, 25]. This is, however, problematic for the determination of the main sugars in wine, glucose and fructose, as the latter is not a reducing sugar (containing a ketone group instead of an aldehyde function) and is therefore not reactive towards most common derivatization strategies based on reductive amination. A simpler alternative is the use of indirect photometric detection, including the commonly used indirect absorbance detection (IAD) [26-33]. In this method, an UV absorbing co-ion is added to the background electrolyte (BGE) and detection is achieved when the analyte displaces the co-ion, resulting in a drop in the background absorbance. In order to separate the sugars under zone electrophoretic conditions, they have to be charged, which limits the useful pH range to > 12, as these compounds are only very weakly acidic. Methods based on this principle have successfully been used to analyse carbohydrates in some food products [28, 32], although routine applications have yet to be demonstrated. Direct UV detection using complexation of sugars has limited applicability for real samples [34, 35].

In this chapter the evaluation of several of the mentioned analytical methods for the routine analysis of carbohydrates in wine samples is discussed. Important criteria were simplicity and speed (minimum sample preparation), reliability (reproducibility) and sensitivity (suitable for the analysis of dry wines). NP-LC-RI detection is compared to NP-LC-ELSD, and to two CE-IAD methods.

6.2 Experimental

6.2.1 Materials

HPLC grade acetonitrile, 1-naphthylacetic acid (NAA), cetyltrimethylammonium bromide (CTAB) and the sugar standards were from Sigma-Aldrich (Atlasville, South Africa). Formic acid (100%) and pyridinedicarboxylic acid (PDC) were from Acros (Geel, Belgium), while ethyl acetate was from Merck (Darmstadt, Germany). LC mobile phases and wine samples were filtered through 0.45 mm HV filters before use (Millipore Corporation, Bedford, MA). The styrene-divinylbenzene SPE cartridges (Strata SDB-L, 3 mL, 500 mg phase) were from Phenomenex (Torrance, CA, USA). Sodium azide (Sigma) was added to all CE standard solutions. Wine samples were purchased from local stores and, if not analysed directly, transferred under nitrogen to completely filled amber bottles to ensure their preservation.

6.2.2 Instrumentation

LC-RI: Analyses were performed on an HP 1100 LC system equipped with quaternary pump, on-line degasser, autosampler, column heater and refractive index detector (Agilent Technologies, Waldbronn, Germany). Instrument control and data analysis was performed using Chemstation software (Agilent). An Asahipak NH2P polyamine-bonded polymeric gel column was used (250 × 4.6 mm i.d., from Shodex, Tokyo, Japan). The mobile phase was 70% acetonitrile (pre-mixed and degassed), used at a flow rate of 1 $mL \cdot min^{-1}$. The optical temperature of the RI was set to 35°C, and the column was thermostatted to 30°C. 25 µL was injected.

LC-ELSD: These analyses were performed on a modular system consisting of a Waters 510 pump equipped with a U6K injector (Waters, Milford, MA, USA), an evaporative light scattering detector (500 ELSD) from Alltech (Deerfield, IL), and an HP 3396 integrator from Agilent Technologies. A Spherisorb NH2 column (25 cm × 4.6 mm i.d., 5 µm

particles) from Waters was used for the sugar analysis with a mobile phase consisting of 87% acetonitrile in water. The flow rate was 1.1 $mL \cdot min^{-1}$, and the injection volume 10 µL. The ELSD settings were as follows: nebulizer gas flow 2.74 $L \cdot min^{-1}$ and drift tube temperature 85°C.

CE: Experiments and data analyses were performed on a HP3D CE capillary electrophoresis system equipped with diode array detection from Agilent. Bare fused silica capillaries (Composite Metal Services Ltd., Worcester, UK) with an internal diameter of 50 µm and a total length of 64.5 cm (56 cm to the detector) were used. Injection was performed by applying 50 mbar pressure for 2 s, followed by the injection of a buffer plug (50 mbar, 2s). Between runs the capillary was flushed for 4 minutes with buffer, followed by a voltage conditioning step (-15 kV for 1 minute). The separation voltage was ramped from 0 to –13 kV in 0.5 minutes. Indirect UV detection was performed at 350 nm with a reference wavelength of 220 nm. The capillary temperature was set at 15 °C.

6.2.3 SPE sample clean-up

The SPE procedure for LC-ELSD analysis has been described elsewhere [36]. Briefly, the Strata SDB-L cartridges were conditioned with 3 mL each of ethyl acetate, methanol and water (pH 2.5). The pH of the wine sample was adjusted to 2.5 with 6 M HCl, prior to loading of 1 mL onto the cartridge. The organic acids and sugars were removed with 4 × 1 mL of 20 mM sulphuric acid. This combined eluent (5 mL) is used for analysis of sugars. Recoveries as calculated for an artificial wine sample were 106.5 and 91.1 % for fructose and glucose, respectively [36].

6.2.4 Calibration

For LC and CE analyses external calibration of fructose and glucose was performed using standard solutions in 80 % acetonitrile (LC) and water (CE). The calibration graphs were constructed over the range 250-5000 ppm (5 levels, triplicate injections).

6.3 Results and Discussion

6.3.1 NP-LC-RI

NP-LC methods are frequently used for the routine determination of carbohydrates [10, 17-21, 37]. These methodologies are based on elution of sugars using aqueous organic solvents from aminopropyl- [10, 18, 20, 21, 37], cyano- [19] or diol-bonded silica columns [38]. RI detectors are prevalently used in these analyses, as most established HPLC detectors are incapable of detecting underivatized carbohydrates under the operational conditions. The main advantages of these methods are robustness and simplicity, and they are especially suited to samples containing sugars at high concentrations, where dilution can often be performed to reduce interference from other sample components. The combination of NP-LC with RI detection was evaluated as a routine analytical method for wine carbohydrate analysis.

From initial experiments employing a Waters NH2 column (250 × 4.6 mm i.d., 5 μm particles) and aqueous acetonitrile as mobile phase it was evident that the sensitivity of the method rendered it unusable. The noise level decreased with increasing water content of the mobile phase (up to 35%), but retention of the sugars was very poor under these conditions. That the noise originated form the column became clear when replacement of the column by a restriction capillary led to greatly reduced noise. Hydrolysis of these stationary phases under conditions employing acetonitrile-water mobile phases has been shown to occur [38]. Presumably this phenomenon is to blame for the observed noise levels. In addition, problems relating to loss of sugars, resulting from formation of Schiff bases by the reducing sugars, have been documented for amino-bonded columns. A number of tested columns presented similar problems. The best results in terms of noise level and retention were obtained using a Shodex NH2P polyamine-bonded polymeric gel column (250 × 4.6 mm). Glucose and fructose are successfully resolved on this column using a mobile phase consisting of 70 % acetonitrile. Calibration of both compounds was linear over the range 250-5000 ppm and the reproducibility of retention times and peak areas were good. Calibration data and limits of detection for these compounds are presented in table 6.1. The

sensitivity of the method precluded the use of dilution, and wine samples were directly injected. While glucose and fructose could be detected in almost all dry wine samples analysed, the use of direct injection meant that co-elution with other wine constituents occurred in some instances, placing doubt on the accuracy of the method. Sample clean-up by SPE is not a solution to this problem, since in the first place the overlap is probably also due to sugars, while secondly this would introduce unwanted dilution. An example of the direct analysis of a white wine is presented in figure 6.1.

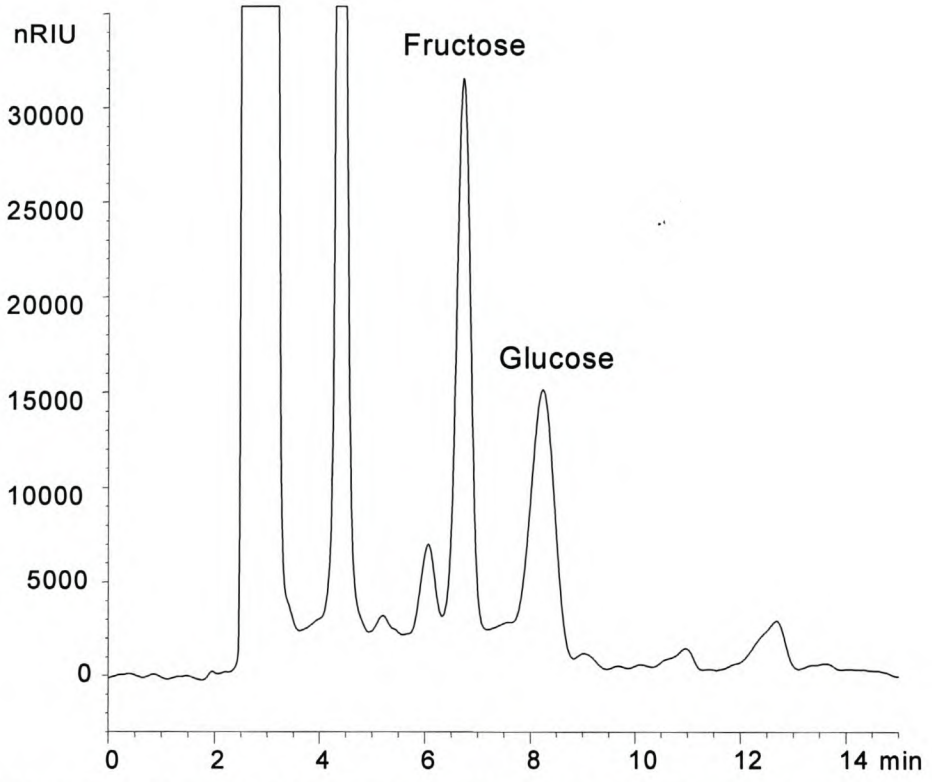

Figure 6.1: Direct NP-LC-RI analysis of South African Chenin Blanc containing 3320 ppm fructose and 2860 ppm glucose.

The NP-LC-RI method presented here was applied to the analysis of ~100 wine samples. Reproducibility of retention times and RI response were acceptable, but limitations in terms of sensitivity and concomitant inaccuracy do detract from the value of the method for routine analysis. Alternative methods were therefore evaluated and compared to the NP-LC-RI method described.

6.3.2 NP-LC-ELSD

The main drawback of HPLC methods for sugar analysis has long been the lack of a suitable detector. RI detection is most often used, but this form of detection suffers from poor sensitivity and, moreover precludes the use of gradient elution needed for the separation of complex mixtures of sugars within an acceptable time. ELSD has been proposed as a useful alternative [10, 21, 37, 38, 39], and in a comparative study this form of detection was found to be superior to RI in sensitivity, while at the same time being amenable to gradient elution [10]. The applicability of NP-LC-ELSD to carbohydrate analysis of wine samples was investigated, and compared to the NP-LC-RI method described above.

In initial experiments the ELSD settings were optimised as specified in the experimental section to produce the optimal signal-to-noise ratio for glucose and fructose. Acceptable separation of wine carbohydrate standards was achieved by isocratic elution using 87% acetonitrile as mobile phase (figure 6.2).

Calibration of fructose and glucose was performed. The response of the ELSD can as yet not be explained perfectly, and depends to a large extent on the physical and chemical properties of the sugars and the mobile phase. A combination of nebulization and light scattering theories predict that the response cannot be truly linear in a significant concentration range. Nevertheless, Macrae et *al.* [37] reported linear responses for glucose and maltose over the range 500-10000 ppm, and Wei et *al.* [21] did likewise for 6 sugars over the range 30-2000 ppm using ELSD detection. On the other hand, in a previous study performed in our group, an exponential response was obtained between 20 and 500 ppm [40]. Similar results were obtained in this study. Details on the calibration equations and LOD are presented in table 6.1.

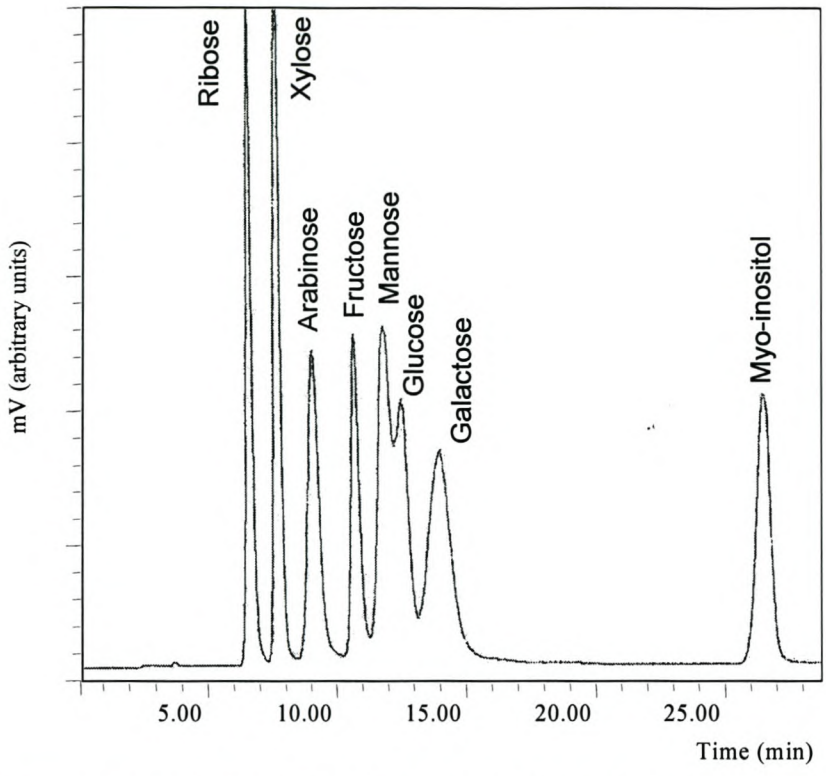

Figure 6.2: NP-LC-ELSD analysis of carbohydrate standards (500 ppm fructose and glucose, 700 ppm xylose and myo-inositol, 900 ppm arabinose, 1000 ppm mannose and ribose, 1100 ppm galactose).

Although non-linear in response, good correlation coefficients were obtained for calibration graphs, and sensitivity of the ELSD is sufficient for the purpose of carbohydrate analysis in wine. In fact, the dilution of wine samples during the SPE clean-up procedure did not present problems in terms of detection of sugars even in dry wines, and clean chromatograms were obtained for SPE samples (figure 6.3).

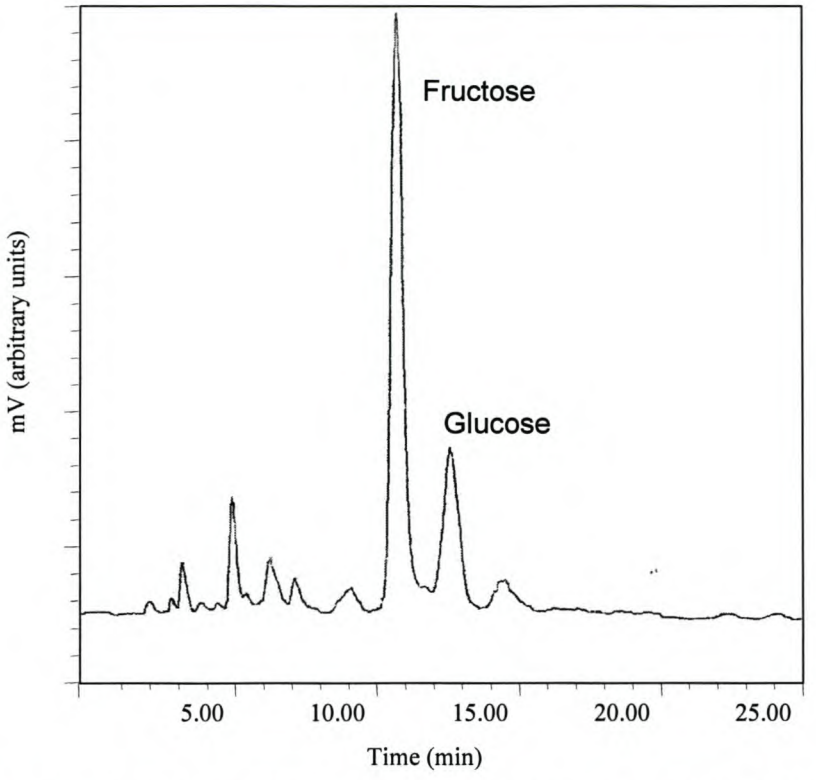

Figure 6.3: NP-LC-ELSD of an SPE sample of a South African Chardonnay containing 1630 ppm fructose and 980 ppm glucose (1:4 dilution).

Although in principle NP-LC-ELSD has been shown to be suited to carbohydrate analysis of wine samples, and offers increased sensitivity compared to RI detection, certain practical problems were encountered. In the first instance, day-to-day reproducibility of the ELSD response was very poor. This meant that calibration has to be performed at least daily, a situation that is clearly not acceptable for routine purposes. Problems relating to increasing solvent pressure were experienced, presumably caused by partial blockage of the nebulizer, which had to be cleaned (off-line) quite often. Overall, the stability of the system was found to be the greatest drawback, making the method unsuitable for routine operation. Mention has to be made regarding the stability of the aminopropyl columns. After extensive usage, significant decrease in retention of the sugars was observed. This is likely the result of hydrolysis of the stationary phase as described by Herbreteau et *al.* [38]. Retention could be improved by flushing the column with 1% di-ethylenetriamine, followed by water, and finally 50% acetonitrile overnight. However, the column took extremely long to stabilize

after such treatment, as reflected by the constantly decreasing retention times. It is possible that the instability of the columns under the conditions employed is to blame for the poor reproducibility of the ELSD response, as well as blockage of the nebulizer. Previously, increase in the noise level of ELSD response when using an acetonitrile-water gradient has been ascribed to hydrolysis of the stationary phase leading to the elution of minute amounts of non-volatile silica based particles that also contaminate the detector [38]. A promising approach to ensure reproducible retention on these columns is to employ dynamic modification by adding small amounts of alkyl-amines to the mobile phase. Wei et *al.* [21] described an interesting method employing a dynamically modified amino column with ELSD detection. These authors made use of the volatile ethylenediamine as modifier to ensure ELSD-compatibility. This approach might improve the reproducibility of the NP-LC-ELSD method, found lacking in this study. Primarily, the use of a more reliable column is expected to improve these results.

6.3.3 CE-IAD

The use of CE with indirect absorbance detection (IAD) offers an interesting alternative to LC methods, as UV detectors are the default detectors used in CE. Initially, a method based on the use of pyridinedicarboxylic acid (PDC) as BGE was investigated, as this system showed good results for the analysis of carboxylic acids in wine [41] and carbohydrates in food samples and glycoproteins [30-32]. In this method, 0.5 mM cetyltrimethylammonium bromide (CTAB) is added to the BGE to ensure an anodic EOF. When working at elevated pH ($\geq$ 12) the sugars are partially negatively charged and thus migrate with the electro-osmotic flow (EOF) towards the anode, thereby shortening the analysis time. A 50 μm capillary and a working temperature of 15°C were chosen to limit Joule heating and the resulting noise. The use of the diode array detector allowed the selection of a signal wavelength at 350 nm and a reference wavelength at 220 nm, to produce positive peaks (PDC absorbs at 220 nm, but not at 350 nm).

In the first step the pH of the buffer was optimized using a standard mixture of sugars and a BGE consisting of 5 mM PDC. The pH range between 11.9 and 12.3 was evaluated. The

effective mobilities of all carbohydrates increases with pH as a result of more complete ionization (the pKa values of these compounds are ≥ 12). Counterbalancing this effect is a slight reduction in the EOF due to the increase in ionic strength with pH (more NaOH added). The net effect is, however, a decrease in the migration times. Slight differences in the pKa values mean that the mobilities of the sugars increase with varying degrees. The best separation of the 2 main sugars, fructose and glucose, from the remainder is achieved at pH 11.8. The effect of the ionic strength of the BGE was varied between 5 and 25 mM. In CE-IAD it is normal to keep the BGE concentration to a minimum in order to reduce noise. This approach is limited by requirements of resolution and reproducibility. Increase in the concentration of PDC led to significant increase in noise, as well as longer migration times for all the acids. This last effect is the result of a decrease in the magnitude of the EOF as discussed above. Optimal separation of the sugar standards was achieved at pH 11.8 combined with a PDC concentration of 10 mM. Sensitivity of the method was rather poor, with limits of detection (LOD) of ~ 100 ppm for both sugars (table 6.1).

A number of wine samples were analysed to determine the efficacy of the method. An example of the direct analysis of a white wine is presented in figure 6.4, where it is clear that the earlier eluting organic acids are well resolved from the sugars. Although reproducibility of migration times for standard compounds was good, this situation deteriorated significantly when injecting filtered wine. Large variations in migration times were observed, affecting peak assignment and quantitation. It is still unclear what exactly is the cause of these variations, however, dilution with the mobile phase, which is generally used to solve such problems, is not an option because of a lack of sensitivity. The use of various extensive conditioning steps did not alleviate the problem. Also, the pH of the BGE had to be adjusted regularly (presumably dissolved CO_2 originating from the atmosphere is responsible for lowering the pH) in order to ensure reproducible migration times of the standards, while the levels of sugars were below the LOD in certain wines. This method was found to be lacking in reproducibility and sensitivity, making it unsuitable for the routine analysis of wine sugars.

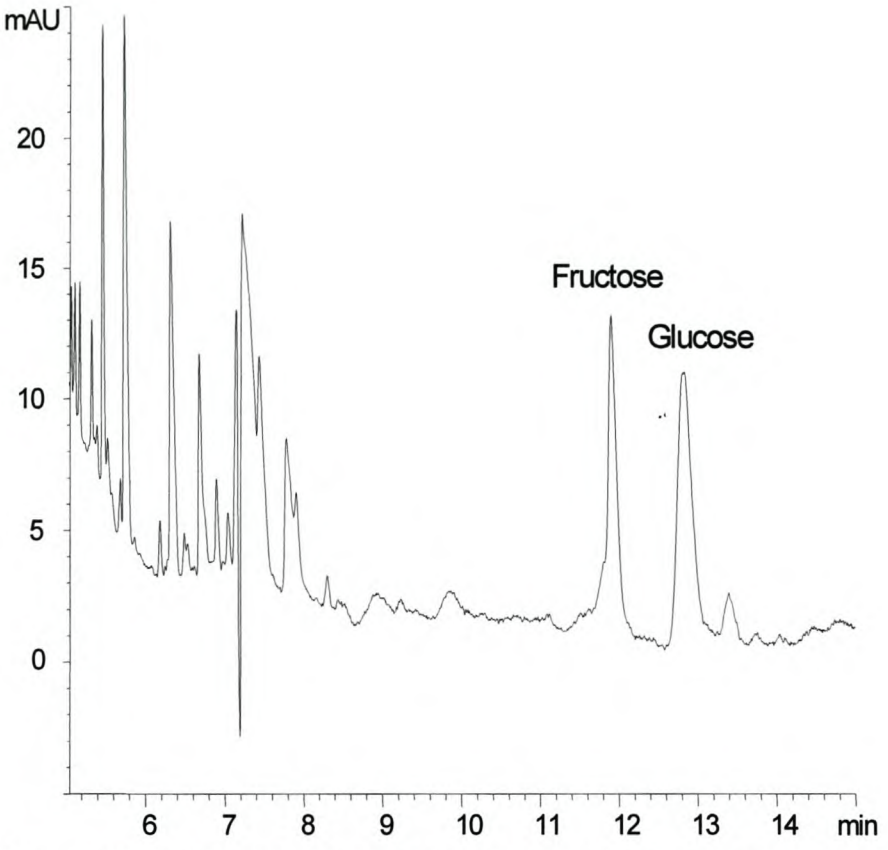

Figure 6.4: CE-IAD analysis of a South African Chardonnay containing 1710 ppm fructose and 1680 ppm glucose (diluted 1:1).

In order to overcome the problems encountered with the PDC method, the use of a different BGE was investigated. Lee et *al.* [28] proposed the use of 1-naphthylacetic acid (NAA) for carbohydrate analysis. This BGE was chosen as alternative because of the following reasons: (1) higher molar absorbtivity (81100 at pH 12, compared to 43680 at pH 5.6 for DPC) should provide improved sensitivity (2) lower mobility (i.e. the mobility is closer to the mobilities of the partially-charged sugars), which should translate into better peak shapes and (3) CTAB is not used in this method to reverse the EOF, and it was thought that reproducibility might improve under these conditions [28, 31]. Optimization of the method was performed in the same manner as for PDC. The best results were obtained at a pH of 11.9 and a BGE concentration of 2 mM. The slightly better sensitivity obtained for the NAA BGE allowed 1:1 dilutions of wine samples, and reproducibility was also improved, presumably as a result of dilution. However, the expected improvement in peak shape was

not observed. Glucose and fructose could not be resolved from the other sugar standards, and eluted close to the EOF peak, rendering this method unsuitable for wine analysis.

Although the applicability of CE-IAD methods has been demonstrated for real samples containing large amounts of sugars, where 50-100 times dilution is often performed, the method is not suited to the determination of sugars at the levels found in dry wines. This is partly because of certain problems inherent to the methodology of CE-IAD analysis of underivatized sugars.

In the first instance, the need to work at pH ~12 limits the usefulness of the method. In the case of negative visualising ions (the situation mostly employed), where weakly acidic buffer systems are used, the analysis is performed outside the buffering range, resulting in poor reproducibility. The excess base added to obtain this high pH also has a very detrimental effect on the separation, primarily in terms of sensitivity (the increase in conductivity leads to Joule heating as well as excessive noise [42]). The sensitivity of the method compares poorly to what can be achieved for other anionic compounds such as the organic acids, as a result of this and a number of other reasons. The degree of displacements of the visualising ion by the analyte, called the transfer ratio (TR), determines the detector response in IAD. The TR is affected by the relative mobilities and charges of the visualising ion and the analyte [26]. Since the visualising ion is completely charged under these conditions, and the sugars are only partially charged, the TR is always low. In addition, the TR is lowered as a result of a competing effect to replace the visualising ion between the analyte and the excess hydroxide ions present at this pH. The mismatch in mobility between analyte and visualising ion also means that poor peak shapes are obtained as a result of electromigration dispersion [42], thereby further increasing the LOD. The relatively poor sensitivity of these methods means that dilution of samples is impossible. The direct injection of complex samples into an un-buffered capillary then leads to poor reproducibility. Finally, the useful pH range is severely limited, making optimisation of the method in the case of complex samples very problematic. The pH has to be $\geq$ 11.8 to ensure sufficient ionisation of the sugars. On the other hand, increase in noise as a results of increased conductivity, as well as a decrease in TR because of excess OH-, limit the pH to a value <12.4. It is evident from the discussion above that the CE-IAD methods mostly employed, and investigated in this study, are unsuitable for the analysis of

wine carbohydrates. The development of suitable derivatization strategies, also allowing the sensitive detection of the non-reducing fructose, possibly in combination with fluorescence detection, might shed new light on this in the future.

Method/Analyte	Range[a]	Equation	R^2	LOD[a]	Advantages	Disadvantages
NP-LC-RI						
Fructose	250-5000	y = 206.x - 1981	1.0000	110	Simplicity,	Poor sensitivity,
Glucose	250-5000	y = 185.x - 2287	1.0000	187	robustness	accuracy, no gradient
NP-LC-ELSD						
Fructose	250-5000	$y = 2708x^{1.311}$	0.9925	19	Sensitvity,	
Glucose	250-5000	$y = 1730x^{1.402}$	0.9929	22	gradient	Poor reproducibility
CE-IAD[b]						
Fructose	250-5000	y = 0.0308.x - 0.478	0.9971	83		Poor reproducibility,
Glucose	250-5000	y = 0.0356.x - 0.208	0.9995	103	Simplicity	sensitivity

[a]Values in ppm (µg/mL)
[b]Details presented for the PDC method

Table 6.1: Figures of merit for each analytical method.

6.4 Conclusion

Three methods have been evaluated for the analysis of carbohydrates in wines. NP-LC-RI is a simple, robust method, but suffers from poor sensitivity. Direct injection of dry wines leads to co-elution of the sugars with other wine components. NP-LC-ELSD offers increased sensitivity, allowing the determination of sugars in wines samples after SPE sample clean-up. However, reproducibility of the method is poor, making the method unsuitable for routine analysis. The instability of the amino-bonded columns used could be at least partially responsible for the poor reproducibility of the ELSD response, as well as the poor sensitivity of the RI. Two CE-IAD methods were found lacking in sensitivity and reproducibility required for these analyses, partially due to the inherent difficulties of their methodology. Although none of the investigated methods were ideal, NP-LC-RI was found the most suited for the routine determination of sugars in wine samples.

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7

Analysis of Phenolic Compounds in Red Wines by LC- and CE-MS*

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7.1 Introduction

The quality of wine depends on many factors. Phenolic compounds are essential constituents because they show marked biological activity, influence sensory properties of wines like bitterness and astringency, and profoundly affect the process of wine ageing [1-4].

Because of ruggedness, sensitivity, and ease of operation, reversed-phase LC with UV or DAD detection is presently the most common technique for the analysis of phenolic compounds in wines [5-11]. Often, UV spectra of phenolic compounds are very similar thereby hampering unambiguous identification. LC-MS, by the introduction of bench-top instrumentation, has evolved to a routine technique that enables collection of significant data on the structures of phenolic compounds in wine [11]

Because of high efficiency and speed of analysis, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) have been used to analyze polyphenols, mostly as standards or in simple extracts [12-15]. CE of wine and wine extracts, using UV or DAD detection, has been reported [7, 10, 16] as an alternative technique to LC for the characterization of phenolic compounds. Electrodriven separation techniques can be hyphenated to mass-selective detection methods, but the performance of CE-MS strongly depends on the separation problem at hand.

In this chapter, a comparison between LC-MS and CE-MS, using commercial instrumentation, for the analysis of phenolic compounds in diethyl ether extracts of red wines is presented.

7.2 Experimental

7.2.1 Chemicals

Diethyl ether was purchased from Fluka AG (Bornem, Belgium). Methanol, isopropanol, and water (Biosolve, Valkenswaard, The Netherlands) were of HPLC-quality. Acetic acid,

ammonium acetate, ammonium tetraborate, and sodium tetraborate were obtained from Sigma-Aldrich (Bornem, Belgium) and ammonia from Ferak (Berlin, Germany).
(-)-Epicatechin, (+)-catechin, 3,4-dihydroxybenzoic acid, and gallic acid were purchased from Sigma-Aldrich (Bornem, Belgium), p-coumaric acid from Acros (Geel, Belgium), and myricetin, quercetin, kaempferol, and caffeic acid from Fluka (Bornem, Belgium). These standards were dissolved in methanol/water 3/1 (*v/v*) at a concentration level of 250 or 500 ppm, depending on the analysis.

7.2.2 Extraction of Phenolic Compounds in Red Wine

A simple enrichment procedure, based on the method described by García-Viguera and Bridle [7], allows extracting and concentrating several phenolic compounds from red wines. This extraction procedure does not provide an extract with all phenolic compounds present in wine, but for the purpose of this study, namely the comparison of LC-MS and CE-MS, diethyl ether extracts give the necessary information. The wines selected for this study were Cabernet Sauvignon (South Africa), Ruby Cabernet (South Africa), Merlot (South Africa), Pinotage (South Africa), Bordeaux (France), and Côtes du Rhône (France). To 50 mL wine, 50 mL water was added and the solution, of which the pH was not adjusted, was extracted with 40 mL diethyl ether. The ether phase was concentrated *in vacuo* (rotavapor) and the residue was redissolved in 2.5 mL methanol/water 1/1 (*v/v*).

7.2.3 High Performance Liquid Chromatography

LC-UV-MS analyses were carried out on a benchtop HP1100 series LC-MSD single quadrupole instrument equipped with a variable wavelength detector (Hewlett Packard, Waldbronn, Germany). An Alltima C18 column, 250 mm x 4.6 mm i.d., 5 µm particle size (Alltech, Lokeren, Belgium), was used. The mobile phase consisted of 1% (*v/v*) acetic acid in water (solvent A) and 1% (*v/v*) acetic acid in methanol (solvent B), and the gradient applied was: 0-25 min: 10-22% B, 25-45 min: 22-50% B, 45-55 min: 50-95% B, 55-60 min: 95% B isocratic, 60-63 min: 95-10% B, 63-66 min: 10% B isocratic. The flow rate was 1 $mL \cdot min^{-1}$ and the analyses were performed at 22°C. The injection volume was 10 µL and the detection wavelength was set at 280 nm. Atmospheric pressure electrospray

ionization (AP-ESI) was carried out in the negative mode. The parameters were: mass range: 80-400 amu; fragmentor voltage: 60, 100 or 150 V; N_2 drying gas flow rate: 12 $L \cdot min^{-1}$; nebulizer pressure: 55 psig; N_2 drying gas temperature: 320°C; capillary voltage: 4000 V.

7.2.4 Capillary Electrophoresis

Analyses were carried out using a Hewlett Packard HP^{3D} Capillary Electrophoresis System with diode array detection (DAD) (Hewlett Packard, Waldbronn, Germany). Separations were performed on bare fused silica capillaries (Composite Metal Services, Worcester, UK). The separation buffers were sodium tetraborate or ammonium tetraborate pH 9.3 or ammonium acetate pH 9.5 (adjusted with ammonia). The capillary was rinsed with water and running buffer before each injection. Injections were performed hydrodynamically. UV-detection was done at 280 nm. CE was coupled to an HP1100 MSD using a prototype CE-MS interface shown in figure 7.1 (Agilent Technologies, Waldbronn, Germany).
The make-up flow used as sheath liquid consisted of methanol/isopropanol 80/20 (*v/v*) and 5 $\mu L \cdot min^{-1}$ was delivered by the HP1100 LC pump operated at a 1/100 split ratio. The parameters were: mass range: 120-400 amu; fragmentor voltage: 70 V; N_2 drying gas flow rate: 7 $L \cdot min^{-1}$; nebulizer pressure: 10 psig; N_2 drying gas temperature: 310°C; capillary voltage: 3500 V.

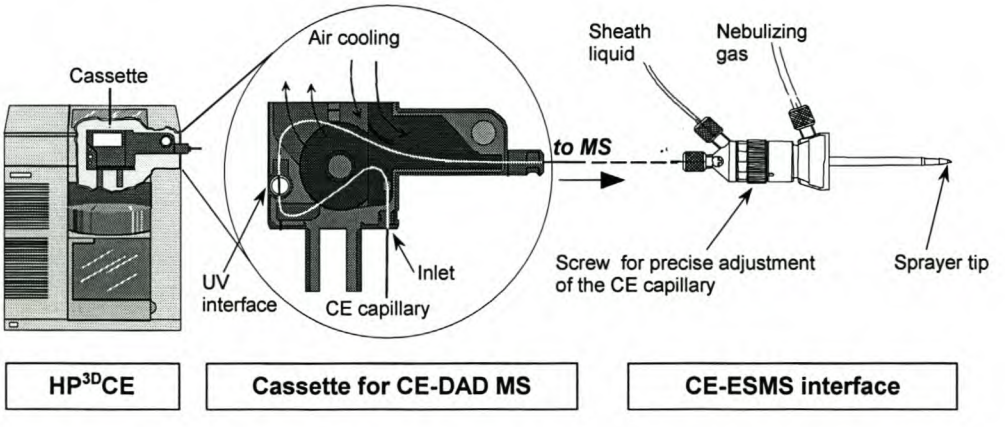


Figure 7.1: General set-up to perform CE-DAD-MS.

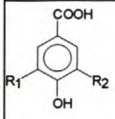
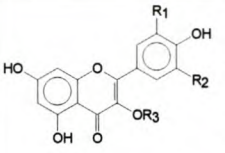
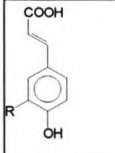
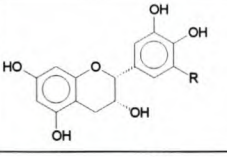
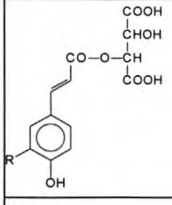
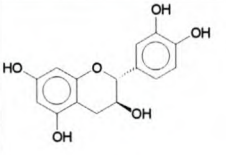
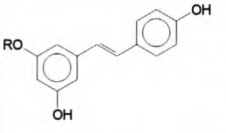
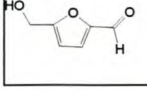
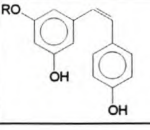
	Compound name (peak number)	*M*		Compound name (peak number)	*M*
	R_1=R_2=H: *p*-hydroxybenzoic acid (**15**)	138		R_1=R_2=R_3=H: kaempferol (**9**)	286
	R_1=H, R_2=OH: 3,4-dihydroxybenzoic acid (**2**)	154		R_1=R_3=H, R_2=OH: quercetin (**8**)	302
	R_1=R_2=OH: gallic acid (**1**)	170		R_1=R_2=OH, R_3=H: myricetin (**7**)	318
	R_1=H, R_2=OCH_3: vanillic acid (**16**)	168		R_1=H, R_2=OH, R_3=rhamnoglucosyl: rutin*(**20**)	610
	R_1=R_2=OCH_3: syringic acid (**17**)	198		R_1=OCH_3, R_2=R_3=H: isorhamnetin (**24**)	316
	R=H: p-coumaric acid (**6**)	164		R=H: (-)-epicatechin (**5**)	290
	R=OH: caffeic acid (**4**)	180		R=OH: (-)-epigallocatechin (**11**)	306
	R=OCH_3: ferulic acid (**19**)	194			
	R=H: coumaroyl tartaric acid (**14**)	296		(+)-catechin (**3**)	290
	R=OH: caffeoyl tartaric acid (**12**)	312			
	4-hydroxyphenethyl alcohol (**13**)	138		R=H: *trans*-resveratrol (**22**)	228
				R=glucosyl: *trans*-polydatin (**18**)	390
	5-hydroxymethylfurfural (**10**)	126		R=H: *cis*-resveratrol (**23**)	228
				R=glucosyl: *cis*-polydatin (**21**)	390

Table 7.1: Compounds identified in wine extracts. Compounds 1-9 were used as standards for method development (* = tentative identification).

7.3 Results and Discussion

7.3.1 LC-UV-MS

LC-UV-MS was optimized using a standard mixture of nine phenolic compounds (figure 7.2). The structures of the standards are given in table 7.1, together with those of the other phenolic compounds identified in the ether extracts of red wines during this study. A

significant difference in response factors between the two detection techniques was noted. Under the applied conditions, MS-detection is more sensitive for the flavonoids (peaks **3**, **5**, **7**, **8**, and **9**) which have UV-absorption maxima around 350 nm, whereas UV-detection shows higher sensitivity for the phenolic acids (peaks **1**, **2**, **4**, and **6**) as a result of the UV-absorption maxima of the latter around 280 nm. Electrospray mass spectroscopy (ESMS) was applied in the negative mode since positive-ion ESMS was less sensitive and did not produce clearly distinctive molecular ions. This phenomenon was also observed by Pérez-Margariño and co-workers [11].

The fragmentor voltage was set at 60, 100 or 150 V. While 60 V resulted in mass spectra with nearly only molecular ion $[M-H]^-$ information, increasing the voltage led to fragmentation via collision-induced dissociation (CID) useful for structure elucidation. A typical fragment of the benzoic acid (**1**, **2**) and cinnamic acid (**4**, **6**) derivatives and the catechins (**3**, **5**) is $[M-45]^-$. This ion results from the loss of a carboxylic group and is already formed at a fragmentor voltage of 100 V.

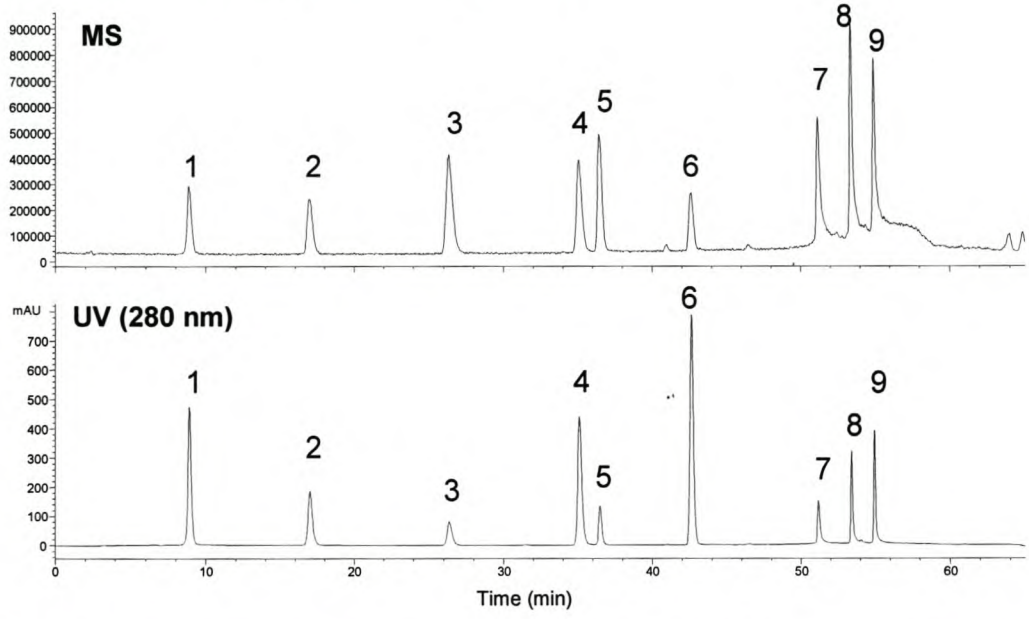


Figure 7.2: HPLC-UV-MS analysis of a standard mixture (250 ppm each). Fragmentor voltage: 100 V. Other experimental conditions: see 'Experimental'. Peak numbering: see table 7.1.

Fragmentation of the flavonols **7** (myricetin) and **8** (quercetin) occurred only at a 150 V fragmentor voltage, while no fragments could be detected for compound **9** (kaempferol) (figure 7.3). This observation is rather conspicuous, while compounds **7** and **8** showed a comparable fragmentation pattern and the structurally related compound **9** did not. The absence of a *meta*-hydroxyl group in the B-ring of kaempferol (figure 7.4) could account for this phenomenon. Fragments *m/z* 151 and 165 for compound **7** and *m/z* 151 and 149 for compound **8** originate from a retro Diels-Alder fission (RDA), resulting in a cleavage of the heterocyclic C-ring [17]. The other pairs of fragments present in the mass spectra (*m/z* 137 and 179 for compound **7** and *m/z* 121 and 179 for compound **8**) are derived from an as yet unclear mechanism, although opening and reclosure of the C-ring concurrent with loss of a carbon atom in this ring, may well proceed.

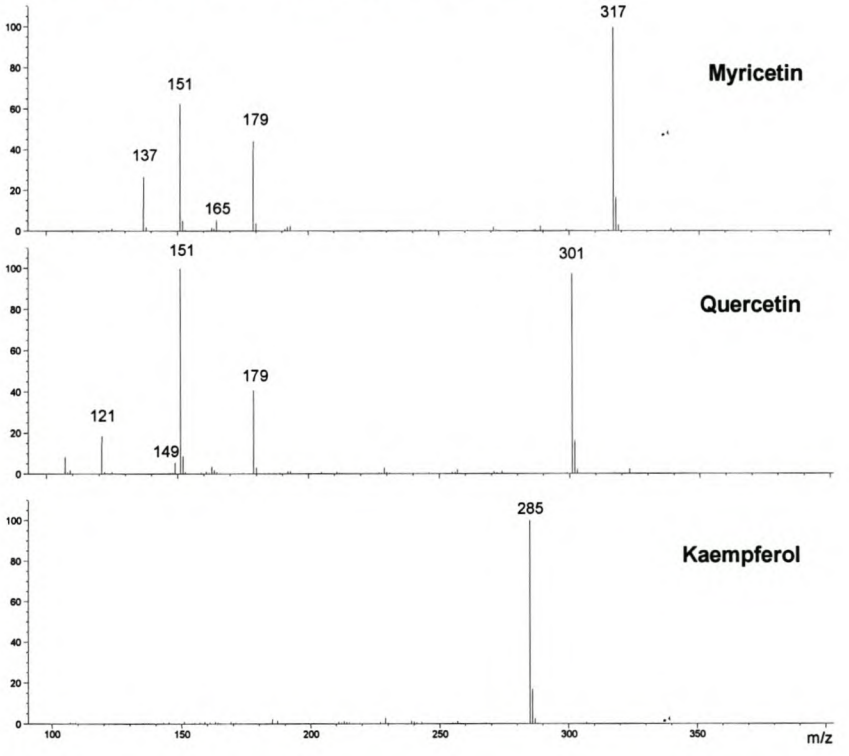


Figure 7.3: Mass spectra of myricetin, quercetin, and kaempferol using a fragmentor voltage at 150 V.

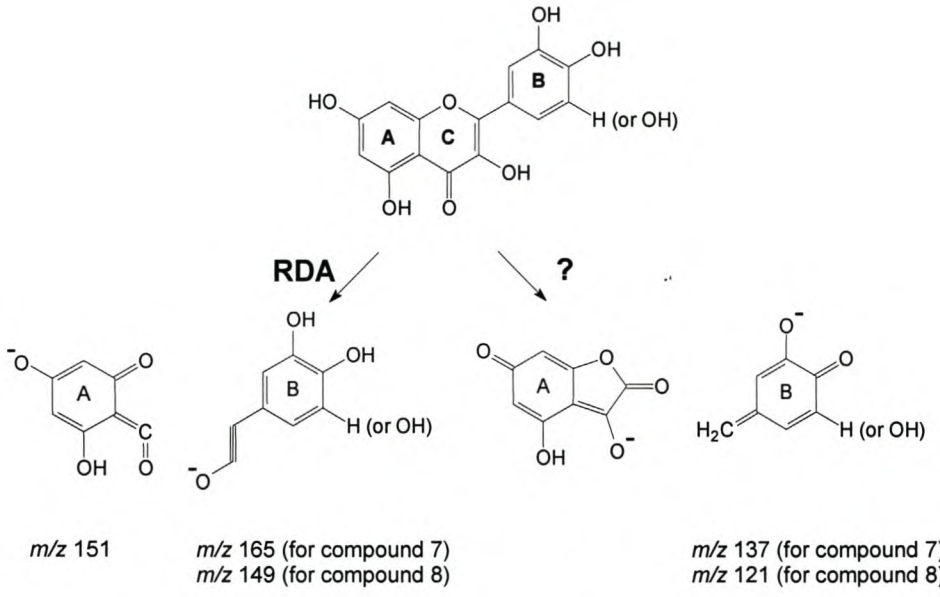


Figure 7.4: Proposed fragmentation for compounds 7 and 8 in electrospray mass spectroscopy with negative ionization.

The experimental conditions used for the standard solution were applied to the analysis of the diethyl ether extracts of red wines. As already mentioned, this extraction procedure does not yield all phenolic compounds present in red wine. More polar solutes are not extracted or exhibit poor extraction efficiencies into diethyl ether compared to more apolar solutes. Solid-phase extraction (SPE) and volume concentration (*via* freeze-drying) could be considered, but due to the complexity of the obtained samples, the diethyl ether extraction procedure was used in this comparative study. The chromatographic profile by UV- and MS-detection for Merlot (South Africa) is shown in figure 7.5. LC-MS analysis allowed structure elucidation of twenty-four constituents based on elution order, mass spectral data and literature data. The elution order is in agreement with the report by Guillén et *al.* [8] and Goldberg et *al.* [9] for the analysis of polyphenols in wines, while the mass spectral data are in accordance to the results from Pérez-Margariño et *al.* [11].

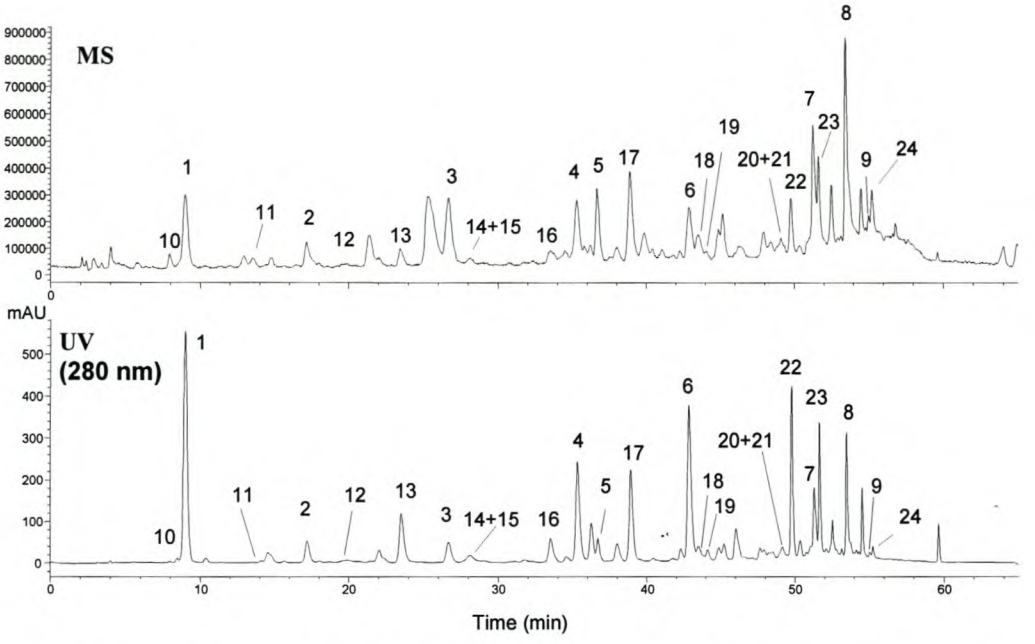


Figure 7.5: HPLC-UV-MS analysis of a red wine diethyl ether extract (Merlot, South Africa). Fragmentor voltage: 100 V. Other experimental conditions: see 'Experimental'. Peak numbering: see table 7.1.

As done for the standards, fragmentor voltages of 60, 100 and 150 V, respectively, were applied and, although not all compounds were resolved, ions could be extracted to obtain a simplified extracted-ion chromatogram (EIC). Using CID, supplementary structural information was collected. The presence of polydatin, a glucoside of resveratrol, could be confirmed. With the fragmentor voltage set at 60 V, only the molecular ion (*m/z* 389) was detected. Both the aglycone (resveratrol, *m/z* 227) and the molecular ion were observed, when the voltage was increased to 150 V. The presence of rutin (compound 20), a diglycoside of quercetin, is based on a tentative identification. Because our study was focused on the low molecular weight phenolic compounds, the mass range of the mass spectrometer was set to a maximum of only 400 amu, while the molecular weight of rutin is 610. At a fragmentor voltage of 150 V, however, the fragment ion *m/z* 301, is the most abundant in the mass spectrum. This *m/z*-value could be indicative for quercetin and taking into account its relative position in the chromatogram, the peak was assigned to rutin.

7.3.2 CE-DAD-MS and CE-MS

CE method development both for the standard solution and the wine extracts prepared according to the method of Garcia-Viguera and Bridle [7] was done without hyphenation to the mass spectrometer. However, in view of the coupling to MS, sodium tetraborate was substituted for ammonium tetraborate. This modification did not significantly influence the CE analysis (figure 7.6).

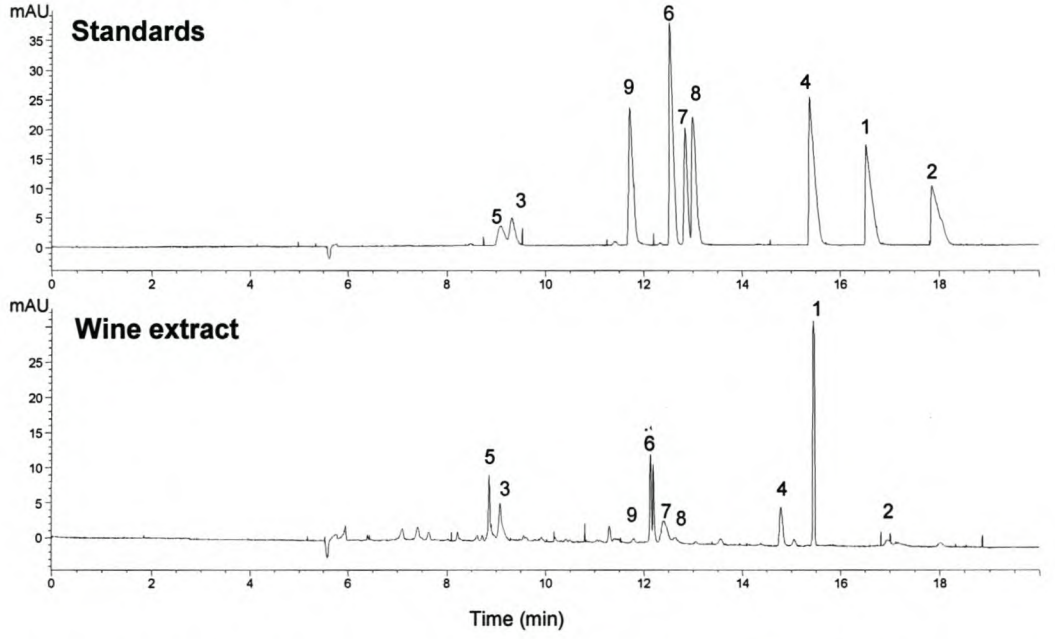


Figure 7.6: CE-DAD analysis of a standard mixture (250 ppm each) and of a red wine diethyl ether extract (Bordeaux, France). Capillary: 50 µm i.d., L = 72.5 cm (l = 64 cm), buffer: 25 mM $(NH_4)_2B_4O_7$ (pH 9.3), V = 22.5 kV, hydrodynamic injection: 50 mbar for 2 s (for standards) and 3 s (for extract), temperature: 30°C. Peak numbering: see table 7.1.

The pH (9.3) of the running buffer allows for complete ionization of all phenolic acids, while the flavonoids (pK_a-values of 9.5-10.5) are only partly ionized. Borate is capable to form complexes with *vic*-diols such as present in some polyphenols. The complexation increases the mass, but also the charge of the complexing solutes and, consequently, their electrophoretic mobility [18, 19]. Accordingly, kaempferol elutes in front of quercetin and myricetin. The large mobility differences between p-coumaric acid and the other (poly)phenolic acids also result from the borate complexation.

The overall sensitivity was low compared with LC-UV. This is a consequence of the small injected volumes and short detector path length in CE compared to HPLC. Variations in migration times and peak shapes, which are apparent from comparison of the standard and wine extract analyses, result from wine matrix effects. DAD detection was not adequate to identify compounds through their UV spectra. Spiking with standard compounds was required and only assumptions could be made on the identity of peaks for which standards were not available. It appears that CE-DAD is not appropriate to achieve a comprehensive qualitative investigation of phenolics in red wines.

The analytical conditions were slightly adapted to perform CE-DAD-MS. The ionic strength of the buffer was decreased from 25 mM to 18.75 mM ammonium tetraborate to obtain a stable electric current and mass signal. This adjustment, conversely, affected the efficiency and the selectivity, since complex formation is directly proportional to the borate concentration [18]. UV-detection was done at 22 cm from the inlet of the capillary, while mass detection was performed at the capillary outlet, which means after 89 cm (figure 7.1). The method was optimized using the standard mixture (figure 7.7). The MS-sensitivity was low and a noisy baseline was observed. A significant efficiency loss was caused by the decrease in ionic strength of the buffer and, apparently, also by the interface.

Peak assignments in CE-MS were based on elution order and mass spectra that were more complex than LC-MS spectra due to the presence of borate in the buffer. Complexation of the borate with the phenols, as well as with the methanol present in the make-up flow, occurred for all compounds containing *vic*-diols. The complexes, detected by an increase in *m/z* of 26, 40, 58 and 72 amu, compared to the molecular ion, were identified as $[M+B(OH)_3-2H_2O-H]^-$, $[M+CH_3OH+B(OH)_3-3H_2O-H]^-$, $[M+CH_3OH+B(OH)_3-2H_2O-H]^-$, and $[M+2CH_3OH+B(OH)_3-3H_2O-H]^-$, respectively. Solutes without a *vic*-diol group did not show this complexation and mass spectra of these compounds are similar to the spectra obtained with LC-MS.

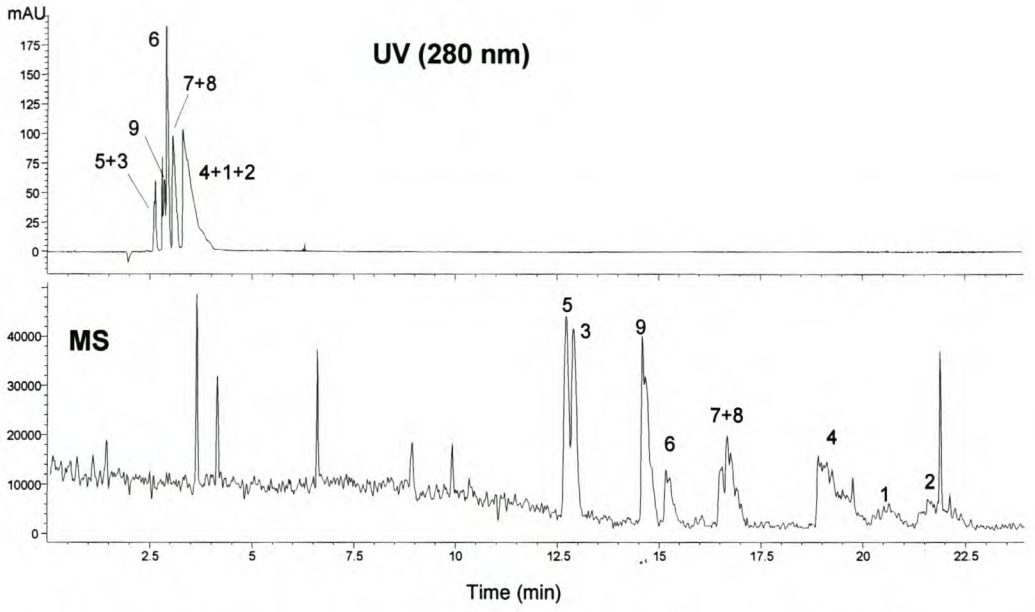


Figure 7.7: CE-DAD-MS analysis of a standard mixture (500 ppm each). Capillary: 50 µm i.d., L = 89 cm (UV-detection after 22 cm), buffer: 18.75 mM $(NH_4)_2B_4O_7$ (pH 9.3), V = 20 kV, hydrodynamic injection: 50 mbar for 2 s, temperature: 25°C. Peak numbering: see table 7.1.

Since the effective length of 22 cm at which UV-detection takes place is too short for sufficient separation, the UV-window of the capillary was cut off shortening the capillary length to 65 cm. A wine extract was analyzed using the same buffer as for the standard mixture (figure 7.8). Due to the low sensitivity and poor resolution, identification of unknowns was not feasible. The presence of compounds differing from the standards could only be confirmed using ion extraction based on the data obtained with LC-UV-MS analyses. The presence of thirteen of the twenty-four constituents detected by LC-UV-MS could be confirmed.

In an attempt to increase the MS-sensitivity, the ionic strength of the buffer was lowered again leading, however, to poor separation. To overcome the sensitivity problem, the use of a volatile buffer like 25 mM ammonium acetate pH 9.5 (adjusted with ammonia) was considered. This electrolyte was successfully used by Aramendia et *al.* to perform CE-ESMS for determination of isoflavone standards [20].

An increased sensitivity was observed for the catechins and the phenolic acids, but the signal and the separation efficiency for the flavonols (peaks **7**, **8**, and **9**) decreased. A

change in elution order of the phenolic acids was also noted. p-coumaric acid elutes after the flavonoids and the elution order of 3,4-dihydroxybenzoic acid and gallic acid is reversed. Overall, the results were not as favorable when compared to the experiments using the borate buffer. Borate complexation seems a prominent factor for successful separation of the phenolic solutes. Furthermore, the presence of only ten compounds could be confirmed in the red wine extract, as opposed to thirteen compounds using the borate buffer.

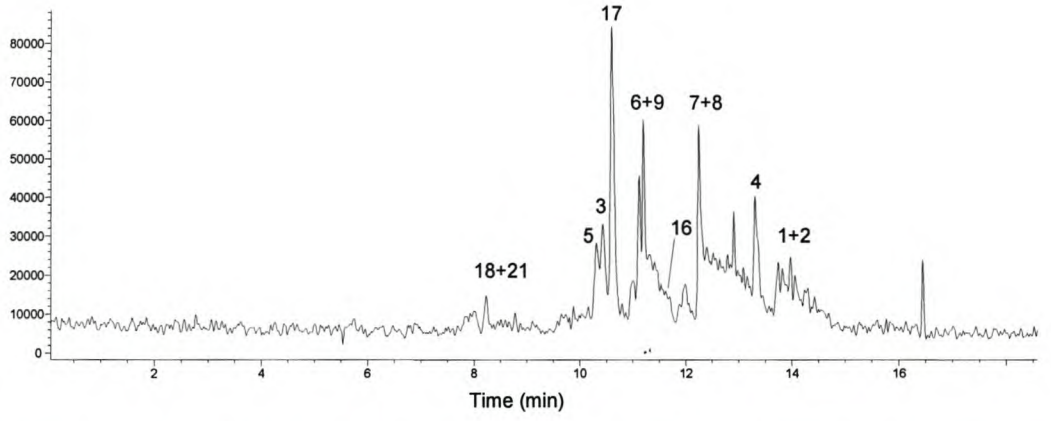


Figure 7.8: CE-MS analysis of a red wine diethyl ether extract (Cabernet Sauvignon, South Africa). Capillary: 50 µm i.d., L = 65 cm, buffer: 18.75 mM $(NH_4)_2B_4O_7$ (pH 9.3), hydrodynamic injection: 50 mbar for 5 s. Other experimental conditions: see Figure 7. Peak numbering: see table 1.

7.3.3 Comparison of extracts from different red wines

CE-MS failed to produce the sensitivity and resolution obtained in LC-MS. LC-MS is therefore, the technique of choice to provide detailed information on the phenolic content of red wines. Figure 7.9 shows the phenolic profiles of the diethyl ether extracts of four different South African red wines using LC-UV-MS. Comparison of the chromatograms reveals significant quantitative differences. p-coumaric acid (peak **6**) is predominant in Cabernet Sauvignon and Ruby Cabernet, while gallic acid (peak **1**) and caffeic acid (peak **4**) are the major constituents in Merlot and Pinotage, respectively. Remarkably, a relatively high amount of the resveratrols (peaks **22** and **23**) clearly differentiates the Merlot wine from the others.

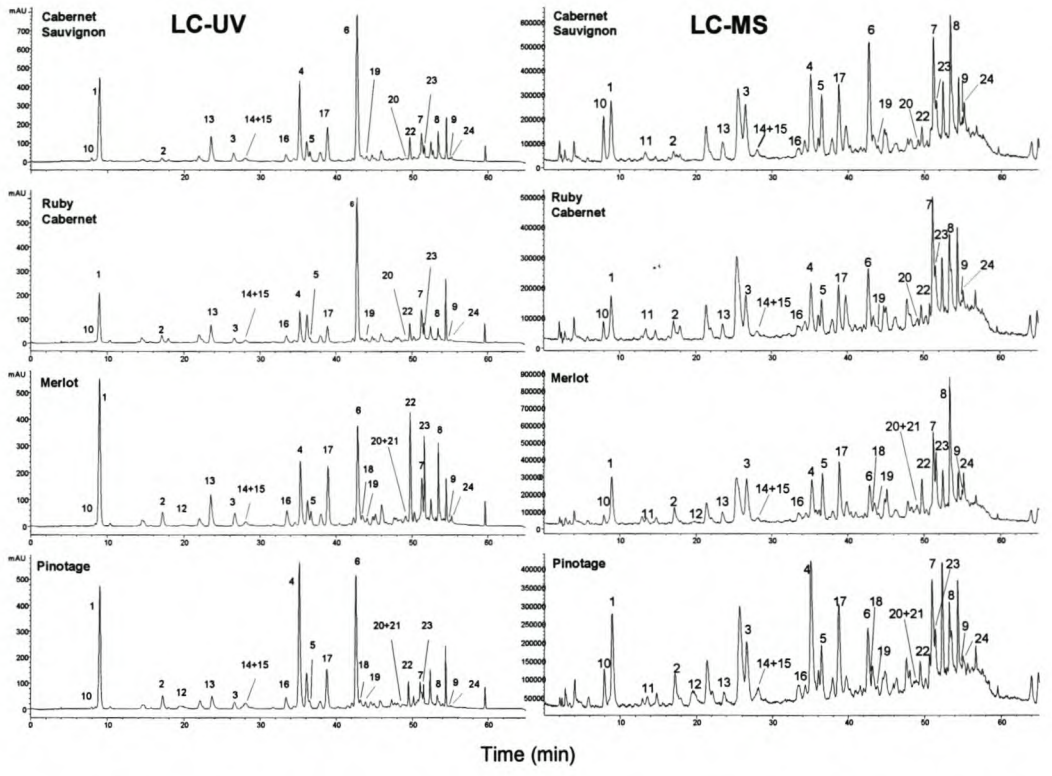


Figure 7.9: LC-UV and LC-MS (with fragmentor voltage 100 V) analyses of diethyl ether extracts of different red wines. Other experimental conditions: see 'Experimental'. Peak numbering: see table 7.1.

7.4 Conclusions

Due to its ruggedness and ease of coupling to mass spectroscopy, reversed-phase HPLC remains the analytical method of choice for the analysis of the phenolic content of complex samples such as wine extracts. Although the extraction procedure used in this work does not yield all phenolic compounds present in red wines, the data clearly shows that LC is far superior to CE for this type of analysis. LC-MS in combination with CID provides structural information on compounds, which cannot be identified from their UV-spectra. CE suffers from low sensitivity and the current state-of-the-art in hyphenation to mass spectroscopy is insufficient to analyze phenols in wine extracts.

7.5 References

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8

Analysis of Wine Phenolics by Direct Injection LC-DAD-IT-MS

8.1 Introduction

Phenolic compounds in wine have been the focus of a lot of research in recent years. They are amongst the non-volatile compounds that are of utmost importance in determining the character of a wine. These compounds are responsible for bitterness, astringency and the colour of red wines and play an important role in the ageing process [1-5], as well as being linked to some of the health benefits associated with drinking wine [6-10].

As a result, exhaustive investigations into the most suitable analysis methods for these compounds have been performed. The use of normal phase liquid chromatography (LC) [11, 12] and capillary electrophoresis [13-15] for the analysis of polyphenols has been demonstrated, however, by far the most useful methods employ reversed phase liquid chromatography (RP-LC). In combination with UV detection, this is the method of choice for analysis of wine phenolics [16-26]. The use of diode array detectors (DAD) allows simultaneous monitoring of different wavelengths in order to optimise selectivity and sensitivity, while providing on-line UV spectra capable of facilitating compound identification. While RP-LC-DAD has been shown to be a rugged analysis method for many of these compounds, the complexity of the wine sample and the similarity in UV spectra of related phenolics complicate identification and quantitation. Sample clean-up is often performed prior to LC-UV analysis of wine phenolics. This takes on the form of liquid-liquid extraction [20, 26] and solid phase extraction (SPE) [16-19, 21, 23], with the latter normally preferred because of increased speed and selectivity, improved recoveries and the option of automation. While these methods have been shown to be effective in simplifying wine LC chromatograms, total analysis time is hereby increased. Also, quantitative and reproducible recoveries of the different classes of phenolics are not possible, and loss of unstable analytes may occur during sample preparation.

The coupling of mass selective detectors to liquid chromatographs has provided a promising analytical method in recent years. The use of MS, often in series with UV detection, offers an attractive alternative for the analysis of polyphenols in wine. Together with the proven

resolving power of RP-LC, unambiguous identification of analytes by mass spectra is now possible. Different ionisation methods have been used for LC-MS determination of phenols. For example, ion-spray [27, 28] and particle beam (PB) [29, 30] interfaces have been used to analyse wine phenolics. With the appearance of reliable atmospheric pressure ionisation methods such as atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI), the number of reports dealing with LC-MS analysis of phenols has increased rapidly [31, 32]. APCI in both the positive [33] and negative [34, 35] modes has been applied for determination of various phenolics in grapes, herbs and olive mill wastewater. Pérez-Magariño et *al.* compared APCI and ESI in positive and negative modes for the analysis of phenolic compounds [36], and concluded that the latter ionisation method was more suited to these analyses. While positive ESI provided the best results for the flavan-3-ols, ESI in the negative ionisation mode had to be used for real samples in order to detect phenolic acids. In fact, positive ESI has been shown to be a very effective detection method for the determination of flavonoids [36] and anthocyanins [37, 38] in wine and isoflavone conjugates in red clover [39], as these compounds are readily protonated under the acidic mobile phase conditions employed. However, when analysing complex samples such as wine, negative mode ESI is the preferred ionisation method even when using acidic eluents, as it provides better mass spectra for a range of compounds including phenolic acids, flavonoids, stilbenes, etc. [36, 40-44].

In most of the cited studies, MS detection was only used for identification purposes, and if quantitation is performed, UV data are used. When the quantitative aspects of MS detection were evaluated, only a partial linear response was reported by Cappiello et *al.* [29] for a PB interface, while more recently Domínguez et *al.* [30] were able to reliably quantify resveratrol derivatives in wine using the same interface. However, the limits of detection (LOD) are in the ppm range, close to those obtained by UV detection. Similar results were obtained by Vial et *al.* using APCI in the negative mode [35]. Gamoh et *al.* [40] reported similar sensitivities for UV and negative ESI-MS (full scan mode) when analysing trans-resveratrol in wine. The MS LOD could significantly be improved when working in the selected ion monitoring (SIM) mode. In this way sensitivity and selectivity are enhanced, however, the power of the mass spectrometer as an identification tool for unknown compounds is sacrificed. On the other hand,

if the aim is to obtain a complete fingerprint of the phenolic pattern of wines, the identification of unknown (i.e. non-standard) compounds is essential.

In this study, the applicability of direct injection followed by liquid chromatography-ESI-ion trap MS for the quantitative and qualitative analysis of a wide range of non-coloured phenolics in red wines is evaluated. The ESI-MS results are compared with the UV results. The merits of each detection method for these analyses are discussed and a new combined UV and MS method is proposed for unambiguous and robust identification of polyphenols. The characteristics of the phenolic compounds studied and identified in the wine are given in table 8.1.

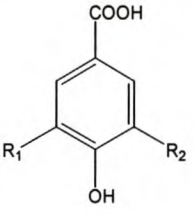
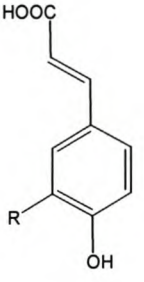
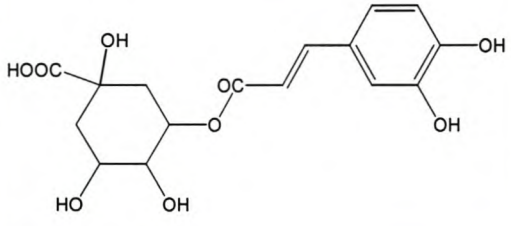
Structure	Name (peak number)	R1/R	R2	R3
	Gallic acid (**1**)	OH	OH	
	Protocatechuic acid (**2**)	H	OH	
	Vanillic acid (**5**)	H	OCH_3	
	Syringic acid (**7**)	OCH_3	OCH_3	
	Caffeic acid (**6**)	OH		
	p-coumaric acid (**9**)	H		
	Ferulic acid (**11**)	OCH_3		
	Chlorogenic acid (**4**)			

Table 8.1: Structures of the phenolic compounds identified in wine.

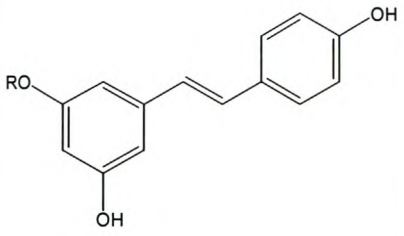
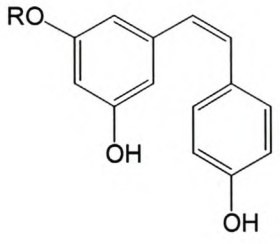
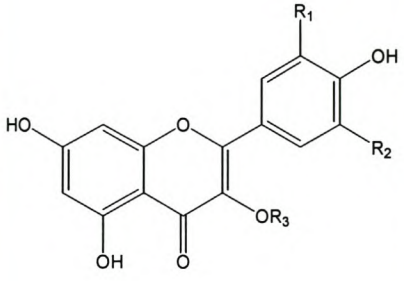
Structure	Name (peak number)	R1/R	R2	R3
	trans-resveratrol (**12**)	H		
	trans-polydatin (**26**)	glucosyl		
	cis-resveratrol (**33**)	H		
	cis-polydatin (**31**)	glucosyl		
	Rutin (**10**)	H	OH	Rhamno-glucosyl
	Myricetin (**32**)	OH	OH	H
	Quercetin (**13**)	OH	H	H
	Kaempferol (**14**)	H	H	H
	Isorhamnetin (**34**)	OCH_3	H	H
	Myricetin-glucoside (**25**)*	OH	OH	glucosyl
	Quercetin-galactoside (**27**)*	OH	H	galactosyl
	Quercetin-glucoside (**28**)*	OH	H	glucosyl
	Kaempferol-glucoside (**29**)*	H	H	glucosyl
	Isorhamnetin-glucoside (**30**)*	OCH_3	H	glucosyl

Table 8.1 (continued): Structures of the phenolic compounds identified in wine (* = tentative identification).

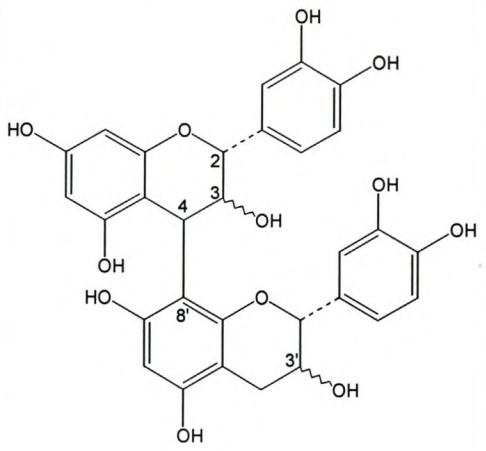
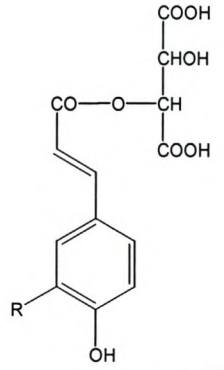
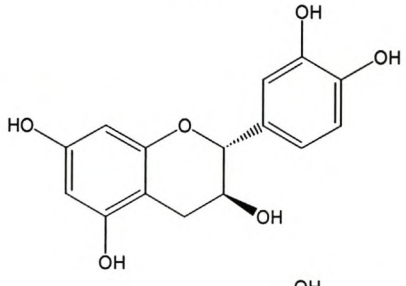
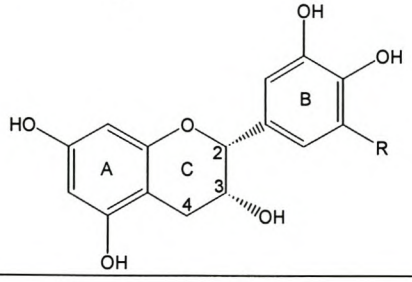
Structure	Name (peak number)	3	3'	4
	Procyanidin B-1 (**19**)	S	R	R
	Procyanidin B-2 (**24**)	S	S	R
	Procyanidin B-3 (**20**)	R	R	S
	Procyanidin B-4 (**22**)	R	S	S
	Caffeoyl-tartaric acid (**21**)	OH		
	Coumaroyl-tartaric acid (**23**)	H		
	(+) Catechin (**3**)			
	(-) Epicatechin (**8**)	H		
	(-) Epigallocatechin (**16**)	OH		

Table 8.1 (continued): Structures of the phenolic compounds identified in wine.

8.2 Experimental

8.2.1 Materials

Analytical grade phenolic standards (gallic acid, protocetachuic acid (3,4-dihydroxybenzoic acid), catechin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, epicatechin, p-coumaric acid, rutin, ferulic acid, trans-resveratrol, quercetin and kaempferol) were purchased from Sigma-Aldrich, Riedel-de Haën (Bornem, Belgium), Acros (Geel, Belgium) and Merck (Darmstadt, Germany). HPLC grade acetonitrile, methanol and acetic acid were from Sigma. Wine samples were purchased from local stores and analysed directly after opening and filtering (0.45 μm).

8.2.2 Instrumentation

LC-DAD-MS analyses were performed on an 1100 LC equipped with a diode array detector and an LC-MSD-Trap with an electrospray ionisation (ESI) source from Agilent (Waldbronn, Germany). The instrument and UV data were controlled by Chemstation software (Agilent), while MS data analysis was performed using DataAnalysis software from Bruker Daltonics (Bremen, Germany).

A Luna C18 column (25 cm × 4.6 mm i.d., 5 μm particles) was used for all analyses (Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) 2% acetic acid in water, and (B) 0.5% acetic acid in 50/50 acetonitrile/water. The following gradient was used: 5% B isocratic for 5 minutes, 5 to 15% B in 1 minute, 15 to 40% B in 24 minutes, 40-55% B in 20 minutes, 55-100% B in 5 minutes, 100% B for 15 minutes, before returning to the initial conditions. The injection volume was 50 μL and the column temperature 25 °C. A flow rate of 1 $mL \cdot min^{-1}$ was used, and detection was performed at 280, 315 and 370 nm, for the detection of flavanols, cinnamic acids and flavonols, respectively. UV spectra over the range 200-600

nm were recorded. Calibration samples containing 0.5, 1, 5, 25 and 50 $mg \cdot mL^{-1}$ (ppm) of all 14 standards were prepared in 40% methanol.

Negative ESI spectra were obtained employing the following conditions: nitrogen as drying gas at 300 °C and a flow rate of 12 $L \cdot min^{-1}$, and a capillary voltage of 3000 V. The scan range was 100-900 m/z. Wine components were identified by comparison of retention time, UV- and mass spectra with standards, and for those not available with data reported in the literature.

8.3 Results and Discussion

8.3.1 Evaluation of the LC-DAD-MS Method

The method used was based on previous work ([42] and de Villiers et *al.*, submitted 2003), and is capable of separating all 14 standard compounds within an acceptable time. Figure 8.1 presents the UV- and total ion current (TIC) chromatograms obtained for the standards. Reproducibility was determined by injecting the same standard solution (25 ppm of all compounds) in triplicate, between wine samples, over a period of 2 days. The LC method proved to be robust, as reflected by the small relative standard deviations (RSDs) for the retention times (table 8.2). The linearity of both modes of detection was evaluated over the range 1-50 ppm. In the case of UV detection, 370 nm was used as calibration wavelength for flavonols (rutin, quercetin, kaempferol), 315 nm for the cinnamic acid derivatives and stilbenes (caffeic acid, p-coumaric acid, ferulic acid and trans-resveratrol) and 280 nm for the flavanols and benzoic acid derivatives. Calibration graphs and limits of detection (at a signal-to-noise, S/N, ratio of 3) were calculated using Chemstation software. Linear responses were obtained for all 14 standards. LOD's for each compound, measured at the calibration wavelength, are presented in table 8.2, and are more than sufficient to allow quantitation of these compounds in wine samples. The LOD for chlorogenic acid (no. **4**) is slightly higher than for the rest of the phenols due to a relatively poor peak shape. Finally, the reproducibility of the UV peak areas was found to be acceptable (table 8.2).

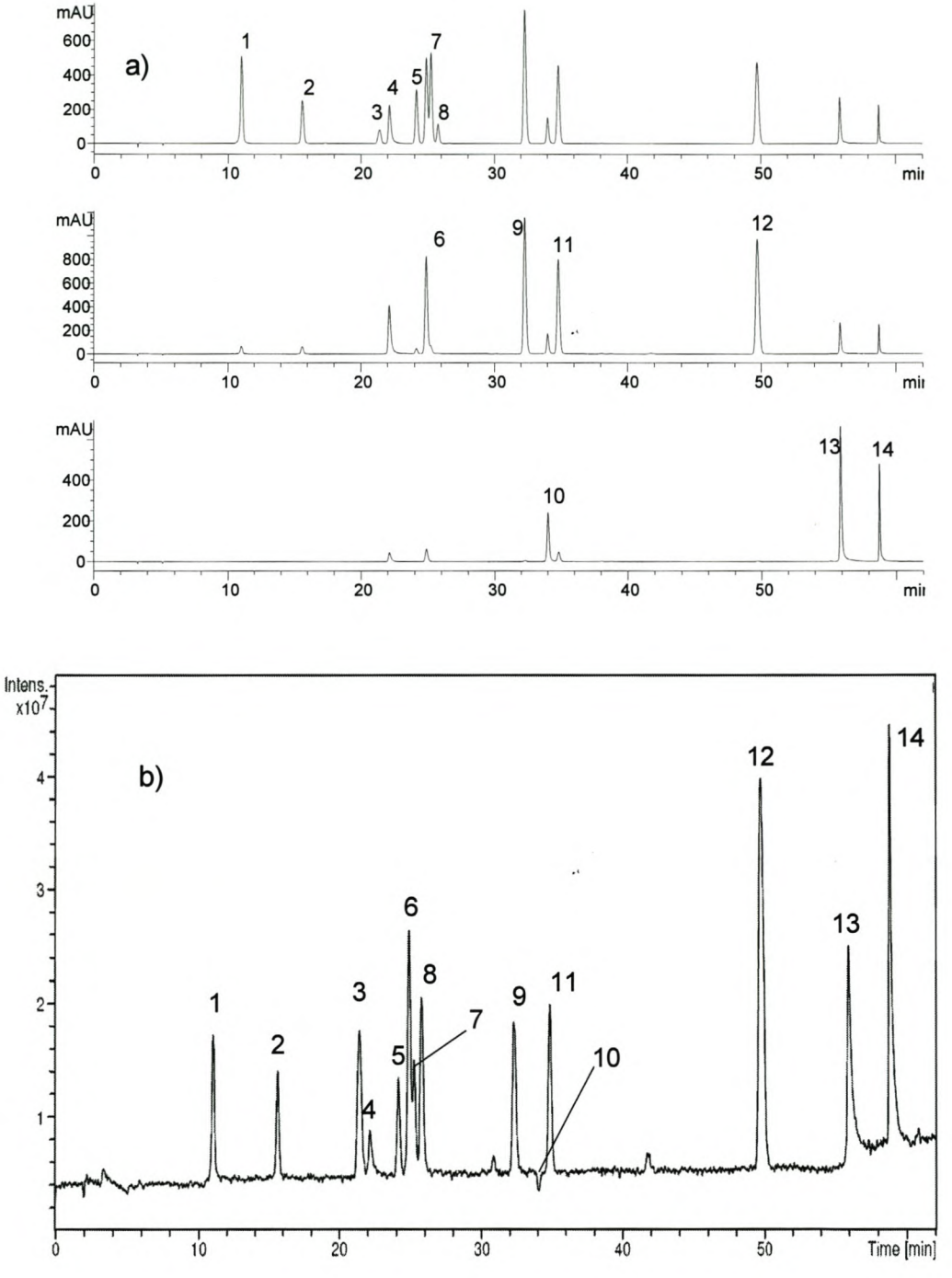


Figure 8.1: RP-LC-UV-MS analysis of standard phenolic compounds: a) UV chromatograms at 280, 315 and 370 nm (from top to bottom); b) TIC chromatogram. For peak identities, refer to table 8.1.

Under the MS condition employed (without fragmentation through collision induced dissociation (CID)), mass spectra with only molecular ion [M-H]- information were obtained (table 8.2). (The mass spectra will be discussed in more detail in the next section). These ions were extracted for each compounds and used to calculate LOD and calibration details. The software used extracted ion chromatograms (EIC) without smoothing to calculate the LOD (at S/N of 3). It is clear from table 8.2 that the sensitivity of MS detection is an order of magnitude lower for certain compounds, compared to UV detection. This is true particularly for certain phenolic acids (compounds **1**, **4**, **6**, **7**, **9** and **11**), but also for rutin (compound **10**). Presumably this is a result of poor ionisation of these compounds under the low pH conditions employed for LC analysis. Acidic conditions are needed to ensure chromatographic retention of the various acids, but in the case of negative ESI-MS analyses, the acid content should be kept at a minimum in order to improve ionisation. On the other hand, for the other flavonols (compounds **13** and **14**) and the flavanols (compounds **3** and **8**), LOD for UV and MS detection were of comparable magnitude. This is in agreement with literature reports [42] using negative ion ESI-MS on a quadrupole instrument. The reason for the poor MS signal for rutin is, however, unclear. The determination of this compound has been reported using mass spectrometry combined with thermospray [45], APCI [46, 47] and ESI [48-51] ionisation techniques, although little quantitative data are given. Mauri et *al.* [49] reported a LOD of 0.3 ppm for rutin using negative ESI, which is half of that obtained in our study.

It has to be noted that these results were obtained in the full scan mode (i.e. monitoring of m/z values between 100 and 900), and that the sensitivity of MS detection can be greatly improved when using the selected ion monitoring (SIM) mode for a quadrupole or in MS^2 or MS/MS mode for an ion trap [40, 47]. This would be an attractive alternative when the analyst is only interested in specific compounds. In this study, however, the aim was also to identify non-standard compounds in wines, necessitating the use of the full scan mode over a wide range.

Linearity of the MS response is acceptable for only a few compounds. The relatively poor reproducibility of MS peak areas (table 8.2) seems to be partially to blame. However, linearity is worse for those compounds showing the highest MS response, indicating that a limited linear response range of MS detection might be another cause of poor quantitation reliability.

UV detection

No.	Compound	m	c	R^2	LOD*	%RSD (Area)	%RSD (Rt)
1	Gallic acid	133.88	28.64	0.9996	0.008	6.1	0.07
2	Protocatechuic acid	70.19	3.77	0.9999	0.019	4.2	0.05
3	Catechin	26.65	2.52	0.9998	0.043	5.6	0.02
4	Chlorogenic acid	122.41	-36.02	0.9998	0.081	4.3	0.12
5	Vanillic acid	83.92	17.94	0.9999	0.013	3.1	0.03
6	Caffeic acid	237.17	-76.40	0.9998	0.005	5.1	0.03
7	Syringic acid	139.12	44.79	0.9998	0.008	2.9	0.03
8	Epicatechin	29.26	7.24	0.9997	0.039	3.8	0.03
9	p-coumaric acid	326.46	87.48	0.9998	0.004	3.8	0.03
10	Rutin	57.30	-0.73	0.9999	0.022	5.6	0.05
11	Ferulic acid	225.94	49.93	0.9999	0.007	3.6	0.03
12	trans-Resveratrol	345.95	83.24	0.9998	0.005	4.7	0.04
13	Quercetin	134.24	-77.94	0.9989	0.028	8.7	0.02
14	Kaempferol	62.05	-29.04	0.9995	0.022	6.2	0.01

MS detection

No.	Compound	m	c	R^2	LOD*	%RSD (Area)
1	Gallic acid	3379051	881987	0.9934	0.203	6.7
2	Protocatechuic acid	2459695	-1755644	0.9934	0.129	8.3
3	Catechin	4389619	21144429	0.9722	0.050	6.2
4	Chlorogenic acid	1290965	2326447	0.9910	1.034	5.3
5	Vanillic acid	1953060	5231697	0.9881	0.115	4.6
6	Caffeic acid	5804706	9269586	0.9942	0.118	4.9
7	Syringic acid	2376977	7196849	0.9871	0.200	3.9
8	Epicatechin	4243546	22914280	0.9564	0.047	7.4
9	p-coumaric acid	3998336	17818135	0.9538	0.053	3.7
10	Rutin	86782	335669	0.9814	0.670	4.9
11	Ferulic acid	3808630	1074473	0.9778	0.184	10.1
12	trans-Resveratrol	13251069	77757006	0.9461	0.049	13.7
13	Quercetin	6624013	47232022	0.9332	0.109	10.5
14	Kaempferol	7447472	70383279	0.8791	0.029	17.2

* Limit of detection (ppm)

Table 8.2: Figures of merit for the LC-UV-MS analysis of phenolic standards (m = slope, c = intercept).

8.3.2 Mass Spectra of Standard Phenolic Compounds

As mentioned above, the pseudo-molecular ion was the base peak in all the mass spectra obtained for the standard phenolic compounds, and little fragmentation was observed under conditions of no CID. Those conditions were used to ensure maximum sensitivity for all compounds, but the use of the ion trap mass spectrometer does allow the study of fragments by multiple stage MS (MS^n) of selected ions.

Table 8.3 presents the fragment m/z values detected under the operating conditions described in the Experimental section, together with applicable references. For the phenolic acids (compounds **1**, **2**, **5-7**, **9** and **11**) the fragment $[M-45]^-$, corresponding to the loss of a carboxylic acid group, was detected in small percentages for both the benzoic- and cinnamic acid derivatives. For vanillic-, syringic- and ferulic acids (numbers **5**, **7** and **11**, respectively), additional fragments for the ion $[M-16]^-$ were detected, resulting from the loss of a methyl group from their respective methoxy substituents [35]. In the case of vanillic and ferulic acids, loss of both methyl- and carboxylic acids groups lead to the fragment $[M-60]^-$ [51]. Syringic acid produces an ion of mass $[M-31]^-$, presumably resulting from the loss of methyl groups from each of its methoxy constituents. The same fragment was reported in [48]. The fragmentation pattern of chlorogenic acid (caffeoylquinic acid, **4**) is consistent with the cleavage of the ester bond, generating [caffeic acid-H]$^-$ (m/z 179) and [quinic acid-H]$^-$ (m/z 191) [46]. The origin of the additional fragment at m/z 176 is unclear. Representative mass spectra of each class are depicted in figure 8.2.

For the flavanols catechin and epicatechin (compounds **3** and **8**) fragments were detected at m/z values of 245, 137 and 125. The first represents the loss of C3 and C4 from ring C, together with the hydroxyl group on C3, leading to $[M-45]^-$ [43, 52, 55]. Retro-Diels-Alder (RDA) fission of ring C leads to the fragment 137 [53], while fragment 125 represents the intact A-ring with 2 phenolic groups, the mechanism of which was proposed by Miketova et *al.* [54].

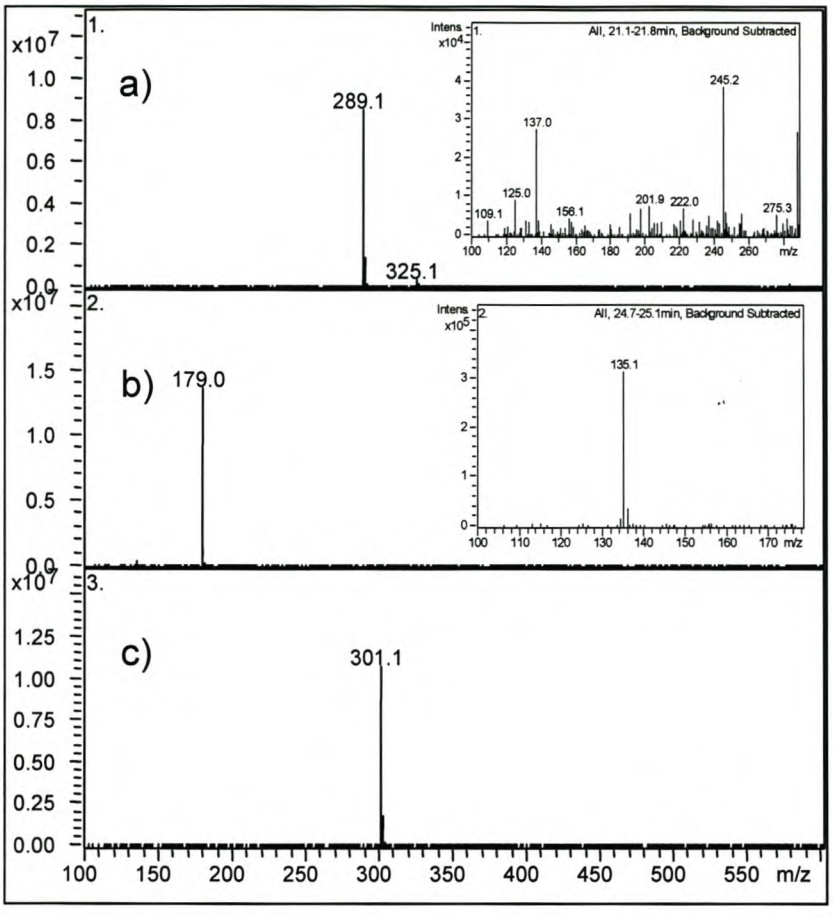


Figure 8.2: Representative mass spectra of a) flavanols (catechin) b) cinnamic acids (caffeic acid) and c) flavonols (quercetin). Inserts: ions detected in MS mode without CID (no ions were detected for quercetin).

In the case of the flavonols, quercetin (**13**) and kaempferol (**14**), no fragmentation occurred using the described analytical conditions. Fragmentation for these compounds has been thoroughly documented using CID [34, 42, 44, 47, 52]. In MS/MS experiments (data not shown) the fragments at m/z 179 and 151 were detected for quercetin, and many fragments, including m/z 151 were detected for kaempferol. Rutin (**10**) did not provide a clear spectrum, but the loss of terminal sugars reported in the literature [42, 47, 51] was observed, although only in MS/MS experiments.

Trans-resveratrol (**12**) also produced no fragments. Deprotonated dimer ions were detected in negligible amounts for some compounds only at the highest calibration levels.

No.	Rt	Compound	$[M-H]^-$	m/z fragment(s) detected (MS mode)	Ref.
1	11.0	Gallic acid	169	125	39,48,32,33
15	13.2	Prodelphinidin 1*	593	289, 243	53,58,63
16	14.6	Epigallocatechin	305	203, 125	39,48,50,51
17	14.7	Prodelphinidin 2*	593	299, 289, 243	53,58,63
2	15.6	Protocatechuic acid	153	109	39,48,32
18	16.7	Prodelphinidin 3*	593	299, 289, 141	53,58,63
19	18.4	Procyanidin B1*	577	175,129	50,53
20	20.0	Procyanidin B3*	577	247,137	50,53
21	21.0	Caffeoyl-tartaric acid	311	179, 149	39,40
3	21.4	Catechin	289	245, 137, 125	39,40
4	22.2	Chlorogenic acid	353	191, 176, 179	43
22	23.8	Procyanidin B4*	577	244,177	50,53
5	24.2	Vanillic acid	167	152, 123, 108	48,33,39
6	24.9	Caffeic acid	179	135	39,32,33,48
7	25.2	Syringic acid	197	182, 167, 153	32,39,48
8	25.8	Epicatechin	289	245, 137, 125	39,40
23	26.9	Coumaroyl-tartaric acid	295	149, 163	39,40
24	29.3	Procyanidin B2*	577	366,287,165	50,53
25	31.1	Myrecitin-3-glucoside*	479	317	40,54
9	32.3	p-coumaric acid	163	119	39,32,33,48
10	34.0	Rutin	609	-	43
26	34.6	trans-polydatin	389	227	39
11	34.8	Ferulic acid	193	178, 149, 134	33,39,48
27	35.3	Quercetin-3-galactoside*	463	-	44
28	35.9	Quercetin-3-glucoside*	463	261,175	30,40,44,54
29	40.4	Kaempferol-glucoside*	447	285,241,217	57
30	40.7	Isorhamnetin-glucoside*	477	287	57
31	42.1	cis-polydatin	389	227	39
32	45.0	Myrecitin	317	-	39,48
12	49.7	trans-Resveratrol	227	-	37
13	55.9	Quercetin	301	-	39,44,41,48,31
33	56.4	cis-resveratrol	227	165	27,39
14	58.8	Kaempferol	285	-	31,39,41,44,48
34	59.0	Isorhamnetin	315	300,151	39,31,44

Table 8.3: Summary of MS data obtained for the analysis of phenolics in wine (* = tentative identification).

8.3.3 Identification of Unknown Compounds in Wine by LC-DAD-MS

Thirteen South African red wines were analysed with the proposed method, with the aim of identifying non-standard phenolics using UV- and mass spectra. Details of the mass spectra and references used are presented in table 8.3.

Epigallocatechin (**16**) was identified on the basis of the molecular ion (m/z 305), the fragment 125 (a characteristic fragment of catechins [54]), UV spectrum closely resembling that of catechin, as well as retention time [42].

Four procyanidins (oligomeric catechins) were detected at m/z 577. This molecular ion indicates a procyanidin dimer consisting of catechin/epicatechin units linked by a C4-C8 or C4-C6 interflavonoid bond [53, 56]. In wine, grape seeds and skins, the presence of the 4 isomers has been reported [17, 57-61]. Identification of these 4 compounds (**19**, **20**, **22** and **24**) is based on the molecular ion, as well as their UV spectra, which are similar to each other and to that of catechin/epicatechin (with maxima at 278 nm as reported [60]). To distinguish between the 4 isomers, it was noted that the elution sequence as reported on reversed phase columns, is: procyanidin B1< procyanidin B3< procyanidin B4< procyanidin B2 [17, 18, 60, 62]. The isomers were tentatively identified accepting this elution order.

In addition, 3 prodelphinidins (oligomeric gallocatechins) were identified in a similar manner. These compounds (named prodelphinidin 1-3, compounds **15**, **17** and **18**) consist of a single catechin/epicatechin unit bound to epigallocatechin as described for the procyanidins. The molecular ion in each case has a m/z value of 593 ($[289+305-H]^-$), and the UV spectrum is similar to those of (epi)catechin, (epi)gallocatechin and the procyanidins. These compounds have been reported in commercial vegetable tanning agents [53] and in grape skins [58]. According to our knowledge, this is only the second time that their presence in wine is confirmed [63]. The retention times relative to the procyanidin dimers are in good agreement with this report.

The mass spectrum of caffeoyl-tartaric acid (**21**) contains only the molecular ion (m/z 311), and deprotonated ions for caffeic acid (m/z 179) [43], and tartaric acid (m/z 149). In addition, the UV spectrum is similar to that of caffeic acid (except for a slight hypsochromic shift), and

the retention time is in accordance with [42]. Similarly, the mass spectrum of coumaroyl-tartaric acid (**23**) contains, apart from the molecular ion, the ionic fragments coumaric acid (m/z 163) [43] and tartaric acid, while the UV spectrum resembles that of p-coumaric acid.

Cis-resveratrol (**33**) was identified by its mass spectrum, displaying the molecular ion at m/z 227 as the base peak, as well as by UV spectrum and retention time, which are in good agreement with [30]. Trans- and cis-polydatin (trans- and cis-resveratrol glucoside, **26** and **31**), displayed similar spectra, with the molecular ions detected at m/z 389 and the aglycones (m/z 227) detected as main fragments. In addition, the UV spectra of each resembled that of its respective aglycone [30], and retention times are in agreement with [30] and [42] (figure 8.3a).

Myricetin (**32**) displayed the characteristic UV spectrum of a flavonol, with maxima at 372, 253 and 224 nm. No fragmentation was observed for this compound, as was the case for the other flavonols in this study, and the molecular ion at 317 m/z is the base peak. Isorhamnetin (**34**) was similarly identified based on its UV spectrum and mass spectrum, in this case containing traces of the $[M-16]^-$ [34, 47] and m/z 151 [34] fragments.

The two flavonol glucosides myrecitin-3-glucoside (**25**, figure 8.3b) and quercetin-3-glucoside (**28**) were identified based on their mass- and UV spectra. The UV spectra of both closely resemble that of rutin (**10**, quercetin-3-rutinoside). This spectrum is characteristic for flavonols, except for a hypsochromic shift of the maxima from 372 nm to 355 nm. The mass spectra in each case showed a clear molecular ion (m/z 479 and 463, respectively), and in the case of myricetin-3-glucoside, the aglycone was also detected at m/z 317. The retention time of these compounds is in accordance with literature reports [33, 43, 47, 57].

Three additional flavonol glucosides were tentatively identified. Quercetin-galactoside (**27**) has an identical UV spectrum to quercetin-glucoside, as well as the same molecular ion as base peak in the mass spectrum. The presence of this compound in wine has not previously been reported, however, the retention time relative to quercetin-glucoside is consistent with [47]. Kaempferol-glucoside (**29**) and isorhamnetin-glucoside (**30**) co-eluted at ~40.4 minutes, and displayed UV spectra very similar to the other flavonol glucosides. Their mass spectra contained the molecular ions at m/z 447 and 477, respectively, with the aglycone kaempferol also detected in the mass spectrum of the former. Once again, their presence in wine has not

been reported, however, their respective retention times are in agreement with literature values [59].

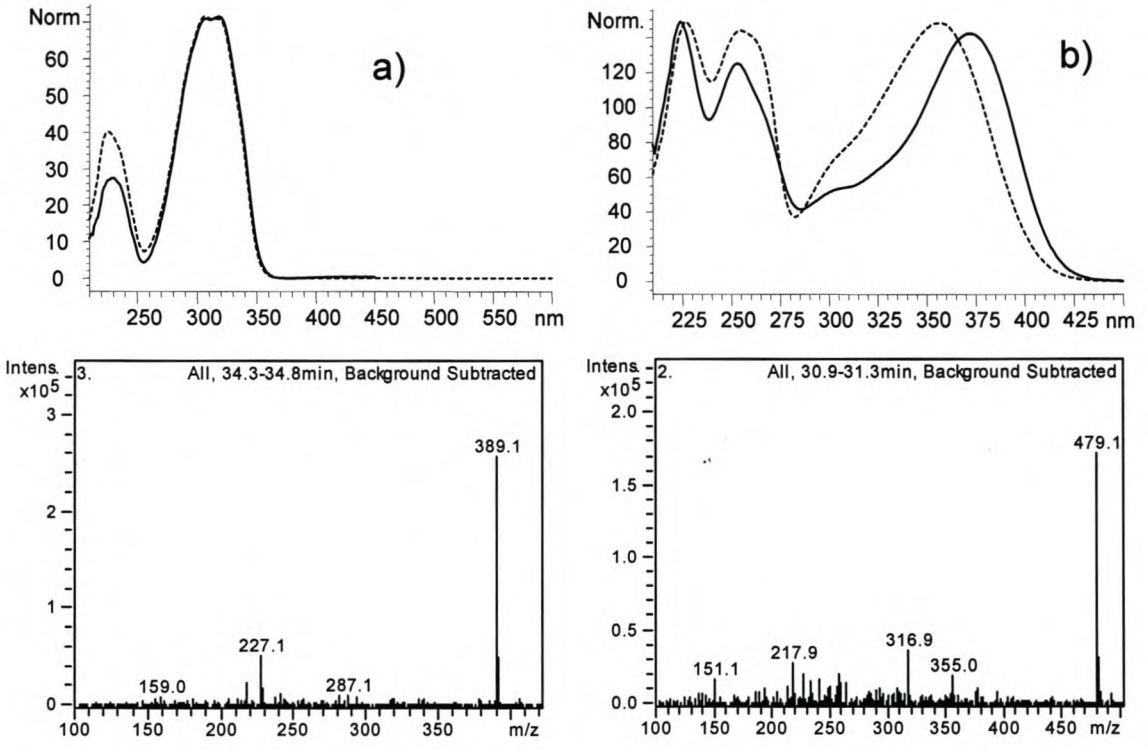


Figure 8.3: Bottom: MS spectra of a) trans-polydatin and b) myricetin-3-glucoside. Top: comparison between UV spectra of a) trans-polydatin (solid line) and trans-resveratrol (dotted line); b) myricetin (solid line) and myricetin-3-glucoside (dotted line).

Typical UV and TIC chromatograms of a South African wine are presented in figure 8.4. For the same wine, extracted ion chromatograms for the procyanidins (m/z 577), cis- and trans polydatin (m/z 389) and cis- and trans resveratrol (m/z 227) are shown in figure 8.5.

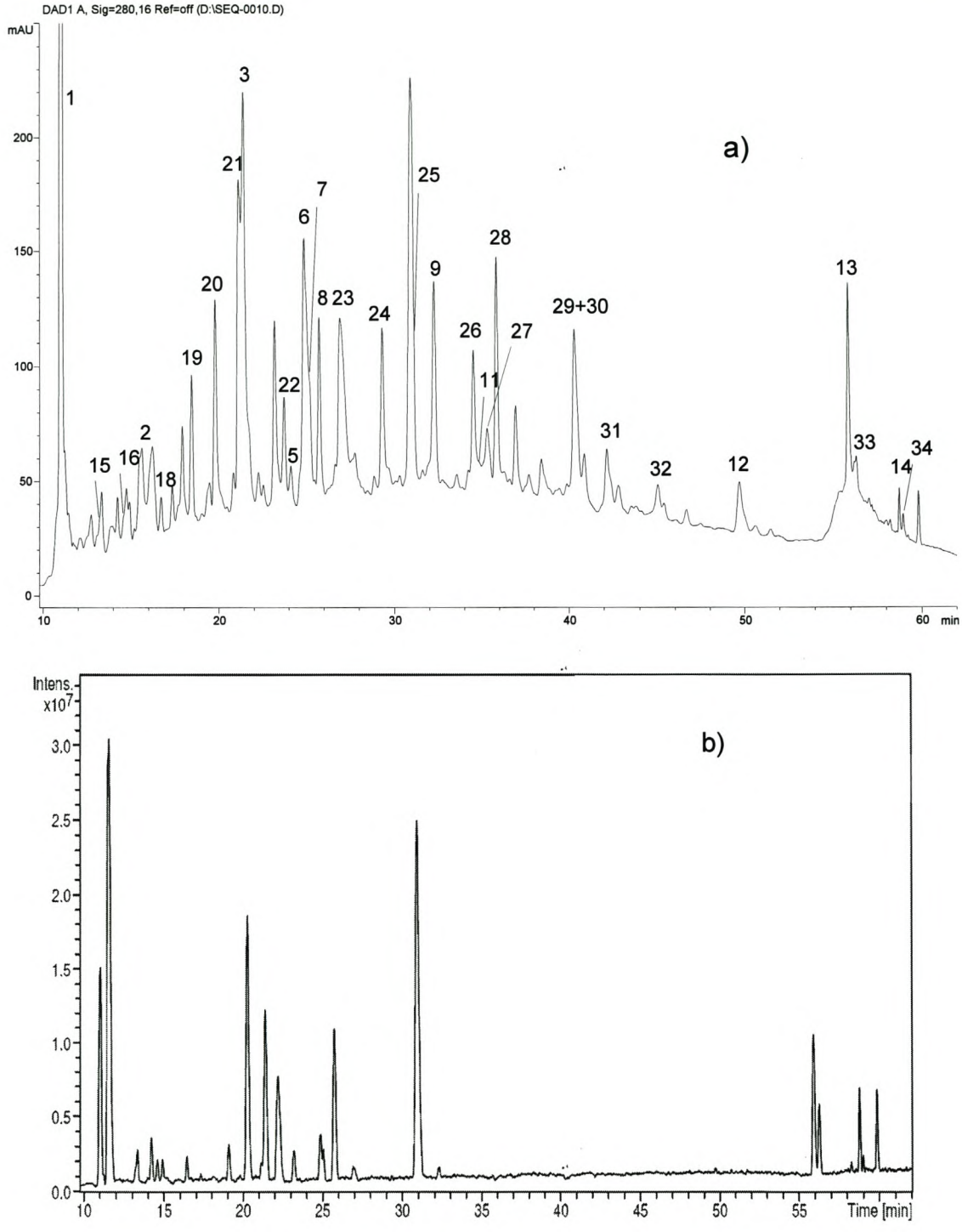


Figure 8.4: RP-LC-UV-MS analysis of South African red wine: a) UV chromatogram (280 nm); b) Base peak chromatogram (m/z 100-600). For peak identification, refer to table 8.2.

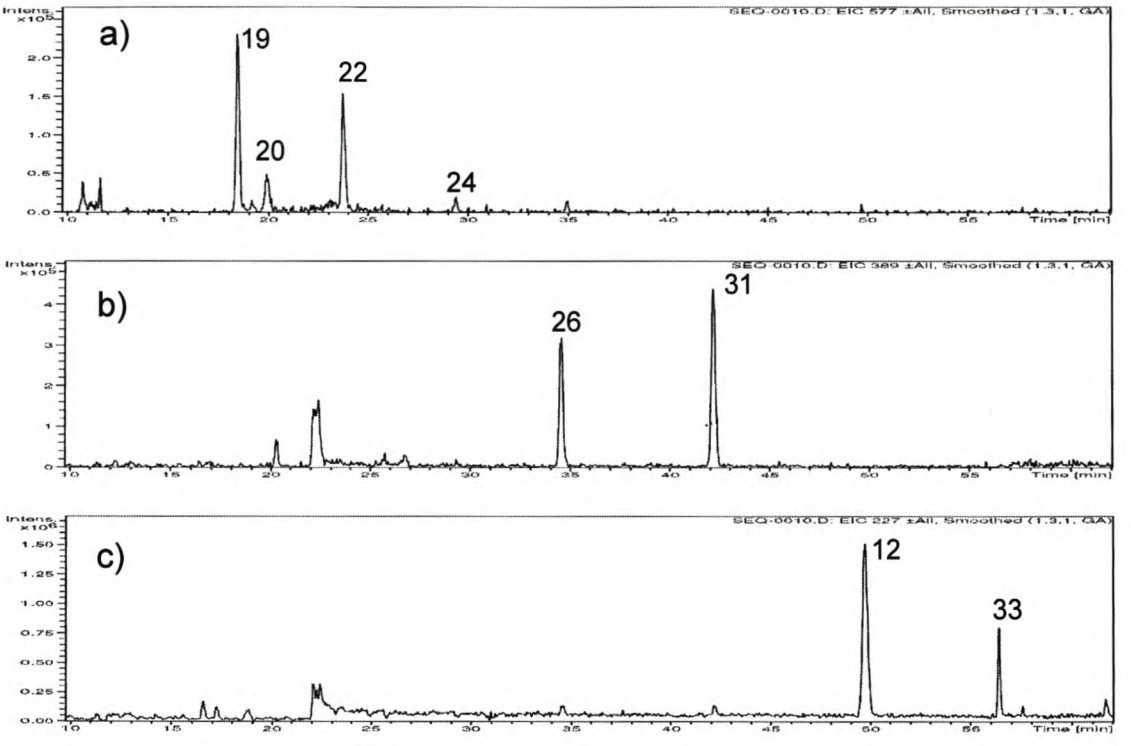


Figure 8.5: Extracted ion chromatograms obtained from the wine analysed in figure 4. a) m/z 577 (procyanidins); b) m/z 389 (polydatins); c) m/z 227 (resveratrols). For peak identification, refer to table 8.2.

8.4 Concluding Remarks

It can be concluded that LC coupled with UV and MS detection is a very powerful separation technique for the direct analysis of wine phenolics, allowing identification of various unknown compounds. Soft ionisation parameters produce mass spectra containing the pseudo-molecular ion as base peak. Combined with the ever-increasing amount of published data on these compounds, characteristic fragmentation patterns can be used for identification purposes. The use of an ion trap instrument allows on-line MS/MS analyses for more fragmentation data. As a result of working in the full scan mode, sensitivity for MS detection is in the same range or

worse than for UV detection. Linearity of the MS instrument was found lacking, and UV detection was more suitable for quantitative purposes. On-line UV spectra in combination with mass spectra greatly facilitate the identification of phenolics. Combination of the two complimentary detection methods with the separation efficacy of reversed phase LC currently represents the state-of-the art in analysis methods for polyphenols in wine. Unfortunately reliable quantitation can only be performed when standards are available.

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9

LC-DAD-MS Analysis of Anthocyanins in Wine

9.1 Introduction

Anthocyanins (anthocyanidin-glycosides) are naturally occurring pigments responsible for the colour of many fruits, including grapes, vegetables and flowers (*anthos* and *kyanos* are the Greek words for flower and blue, respectively). Belonging to the general class of the flavonoids, the anthocyanins are characterised by the cationic flavylium structure, and wine anthocyanins are differentiated based on varying substitution on ring B and the nature of the sugar substituent (table 9.1). The anthocyanins exist in a number of inter-convertible chemical forms, the equilibria of which are pH-dependant. Only at low pH are the red flavylium species predominant, while an increase in pH leads to progressively more of the colourless carbinol-pesudobase and the blue quinoidal base species [1]. In wine media, bleaching by bisulphite, leading to colourless products, and oxidation reactions also take place, ensuring that only a relatively small percentage of the anthocyanins are present in their red flavylium cationic form [2].

The anthocyanins are present in the skins of red grapes, from where they are extracted during maceration, becoming responsible for the purple-red colour of young wines. During ageing, however, the levels of grape anthocyanins rapidly decrease as they react with a variety of other wine constituents [2, 3]. This process, leading to the formation of more stable pigments, is responsible for the change in colour (from purple-red to brick-red) as well as the loss of astringency observed during wine ageing [4]. A number of pathways for these conversions have been proposed and demonstrated. Condensation of anthocyanins with flavanols, either directly [5, 6], or mediated by acetaldehyde [7, 8], has been shown to occur. Cyclo-addition reactions at C4 involving vinyl-phenol [9, 10], pyruvic acid [11-14], acetaldehyde [12] and procyanidin B2 [15] have been reported. These derived pigments are more resistant to increase in pH and bisulphite bleaching, and are orange-red [12, 14, 16]. Furthermore, non-covalent interaction between anthocyanins and other phenolics, known as co-pigmentation, influences the colour of the young red wine, and might be the first step in the formation of pigmented condensed tannins [5, 17].

All the reactions mentioned above contribute to the colour and colour stability of wine, and can influence the organoleptic properties through their effect on the wine tannin structure.

In fact, correlation between wine quality ratings and colour densities (primarily determined by the degree of ionisation of anthocyanins) has been demonstrated for young Australian and French wines [18, 19]. Clearly, the determination of these compounds is an essential part of oenology. Not only can the replacement of the anthocyanins by the more stable derived pigments as the primary colour contributors be studied, this process can also be related to oenological practice [19-22], leading to new insights into the maturation process. In addition, there has been increasing interest in anthocyanins due to their antioxidant capabilities and biological activity [23-25].

Since the report of Wulf and Nagel [26], HPLC has replaced the previously used two-dimensional TLC as most effective separation method for the determination of these compounds. Although spectral methods according to the method of Somers and Evans [27, 28] can be used to estimate the total anthocyanins, polymeric anthocyanins, etc; this does not allow quantification of individual compounds, and moreover leads to overestimation of free anthocyanins [29]. Capillary electrophoresis (CE) has been applied for anthocyanin determination [30-32], but the resolving power and sensitivity needed for wine analysis has not yet been demonstrated. Thus, HPLC in combination with UV detection has become the routine analysis method for the determination of anthocyanins in grapes [26, 33, 34], fruit juices [35] and wine [9-11, 14, 21, 22, 29, 36-38].

With the advent of reliable ionisation sources for coupling LC to mass spectrometry (MS), the number of applications to anthocyanin analysis has increased rapidly. LC-MS was used for the identification of anthocyanins in botanical supplement raw materials [39], fruit [40], grapes [41, 42] and wines [43, 44]. Characterisation of anthocyanins has also been achieved by direct infusion into the MS [45-47] and using MALDI-MS [48, 49]. These instruments are, however, expensive and not commonly available, making LC-UV still the most commonly used analysis method. DAD detection combined with sample clean-up by SPE or liquid-liquid extraction can be used to elucidate the main anthocyanins by their UV spectra upon elution from the column [50]. However, the elution pattern of young wine anthocyanins from a reversed phase column is characteristic enough to allow identification of the main compounds without sample preparation. On the other hand, partly due to the lack of available standards, compounds present in trace amounts have to be identified by on-line MS detection. In the present chapter, the development of an LC-UV method for the

routine analysis of anthocyanins in wine without sample preparation is described. The method was developed with MS compatibility in mind, as identification was performed using mass spectral data.

9.2 Experimental

9.2.1 Materials

HPLC grade acetonitrile was from Sigma-Aldrich (Atlasville, South Africa), formic acid (100%) from Acros (Geel, Belgium), and ethyl acetate from Merck (Darmstadt, Germany). Malvidin-3-glucoside chloride (Oenin chloride) was obtained from Extrasynthese (Genay, France), and dissolved in 1/19/80 HCl/water/methanol. Delphinidin and cyanidin-3-glucoside were kindly donated by the laboratory of Phytopharmacy (Ghent University, Belgium). LC mobile phases and wine samples were filtered through 0.45 μm HV filters before use (Millipore Corporation, Bedford, MA). The styrene-divinylbenzene SPE cartridges (Strata SDB-L, 3 mL, 500 mg phase) were from Phenomenex (Torrance, CA, USA). Wine samples were purchased from local stores and, if not analysed directly, transferred under nitrogen to completely filled amber bottles to ensure their preservation.

9.2.2 Instrumentation

LC-UV: Method development was carried out using UV detection on an Alliance 2690 Separations Module equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). Data analysis was done with Millenium[32] Chromatography Manager software. A Phenomenex Luna C18 column (25 cm × 4.6 mm i.d., 5 μm particles) was used with a mobile phase consisting of (A) 7.5% formic acid, and (B) 7.5% formic acid in acetonitrile. The following optimised gradient was used: 3% B for 1 min, 3-15% B in 11 min, 15-25% B in 12 min, 25-30% B in 4 min, and 30% B for 4 min before returning to the initial conditions. 20 μL was injected and the column was thermostatted to 25 °C. The flow rate

was 1 mL·min^{-1} and detection was performed at 520 nm. UV spectra over the range 200-600 nm were recorded.

LC-MS: LC-MS analyses were performed on a LCQ ion trap mass spectrometer (Thermo Finnigan, San José, CA, USA) equipped with an electrospray interface. A model 325 HPLC pump and UVKON model 735LC single wavelength UV detector set to 520 nm (both from KONTRON Instruments, Watford, UK) was used, together with a Uniflows DG-1310 degasser (Uniflows, Tokyo, Japan). A Phenomenex Luna C18 column (25 cm × 2 mm i.d., 3 μm particles) was used in these analyses, with the same mobile phase and gradient conditions as for the LC-UV analyses and a flow rate of 0.4 mL·min^{-1}. Positive electrospray conditions were optimised by infusion of a solution of delphinidin in phase B, and were as follows: source voltage was 3.8 kV, capillary temperature 225°C, sheath gas and auxiliary gas (both nitrogen) 60 and 20 arbitrary units, respectively. Full scan spectra were recorded over the range 100-1500 m/z. For MS-MS experiments, the molecular ion was isolated in the ion trap, followed by CID at 1.5 V (30%).

9.2.3 SPE Sample Clean-up

In order to obtain clear UV spectra of the anthocyanins, a SPE method was used to remove non-coloured phenolics from a wine sample. The Strata SDB-L cartridges were conditioned with 3 mL each of ethyl acetate, methanol and water (pH 2, adjusted with triflouro-acetic acid). The pH of the wine sample was adjusted to 2 with 6 M HCl prior to loading 2.5 mL onto the cartridge. The polar wine components were removed with 2.5 mL water (pH 2), followed by elution of the non-coloured phenolics with 10 mL ethyl acetate. Wine pigments were then eluted with 2.5 mL of methanol containing 1% HCl and this eluent was directly injected.

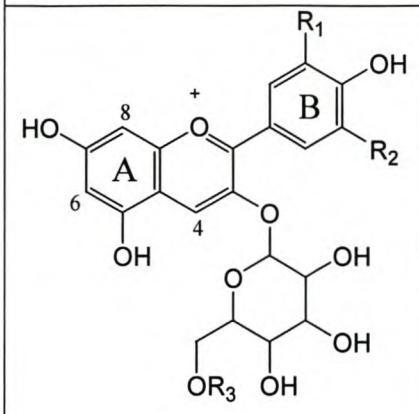
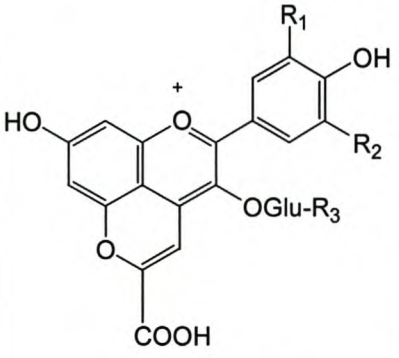
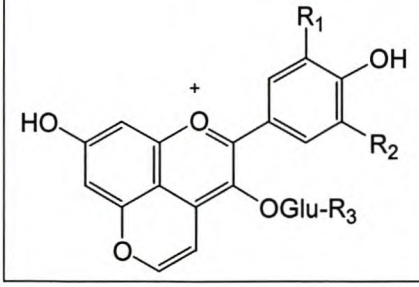
Structure	Name (peak number)	R1	R2	R3
	Dp-3-glucoside (**1**)	OH	OH	H
	Cy-3-glucoside (**2**)	OH	H	H
	Pt-3-glucoside (**3**)	OCH_3	OH	H
	Pe-3-glucoside (**4**)	OCH_3	H	H
	Mv-3-glucoside (**5**)	OCH_3	OCH_3	H
	Dp-acetyl-glucoside (**6**)	OH	OH	acetyl
	Cy-acetyl-glucoside (**7**)	OH	H	acetyl
	Pt-acetyl-glucoside (**8**)	OCH_3	OH	acetyl
	Pe-acetyl-glucoside (**9**)	OCH_3	H	acetyl
	Mv-acetyl-glucoside (**10**)	OCH_3	OCH_3	acetyl
	Dp-coumaroyl-gluc (**11**)	OH	OH	coumaroyl
	Cy-coumaroyl-gluc (**12**)	OH	H	coumaroyl
	Pt-coumaroyl-gluc (**13**)	OCH_3	OH	coumaroyl
	Pe-coumaroyl-gluc (**14**)	OCH_3	H	coumaroyl
	Mv-coumaroyl-gluc (**15**)	OCH_3	OCH_3	coumaroyl
	Mv-coumaroyl-gluc (**28**)	OCH_3	OCH_3	caffeoyl
	Pt-3-glucoside-pyruvic acid[a] (**17**)	OCH_3	OH	H
	Pe-3-glucoside-pyruvic acid[a] (**18**)	OCH_3	H	H
	vitisin A[a] (**19**)	OCH_3	OCH_3	H
	acetylvitisin A[a] (**23**)	OCH_3	OCH_3	acetyl
	coumaroylvitisin A[a] (**27**)	OCH_3	OCH_3	coumaroyl
	vitisin B (**20**)	OCH_3	OCH_3	H
	acetylvitisin B (**25**)	OCH_3	OCH_3	acetyl
	coumaroylvitisin B (**29**)	OCH_3	OCH_3	coumaroyl

Table 9.1: Structures of the pigments identified in wine. Key: Dp = delphinidin, Cy = cyanidin, Pt = petunidin, Pe = peonidin, Mv = malvidin, gluc= glucose. [a] Structure as proposed by Fulcrand et *al.* [13].

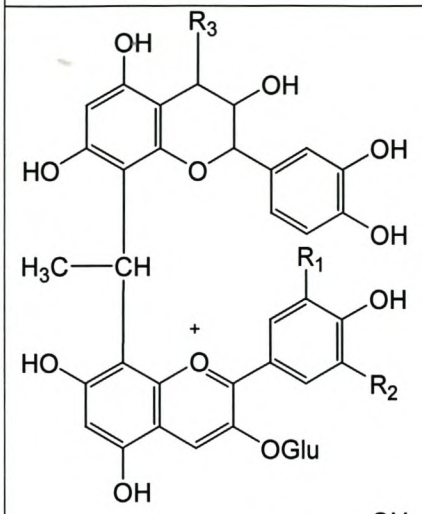
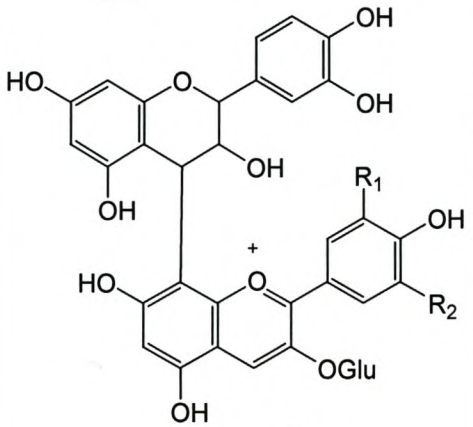
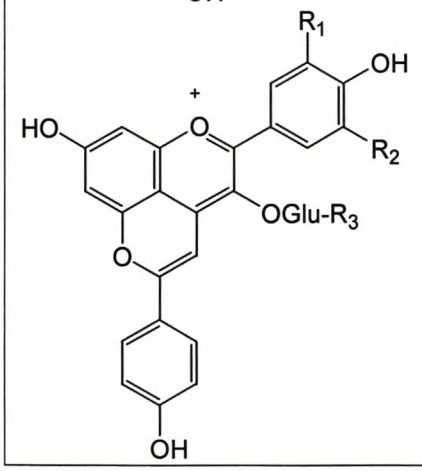
Structure	Name (peak number)	R1	R2	R3
	Mv-glucoside-ethyl-(epi)catechin (**22**, **24**, **26**)	OCH_3	OCH_3	H
	Mv-glucoside-ethyl-(epi)catechin-unknown (**21**)	OCH_3	OCH_3	unknown
	Mv-glucoside-(epi)catechin (**16**)	OCH_3	OCH_3	
	Pigment A (**30**)	OCH_3	OCH_3	H
	Pigment A (**31**)	OCH_3	OCH_3	coumaroyl

Table 9.1 (continued): Structures of the pigments identified in wine. Key: Dp = delphinidin, Cy = cyanidin, Pt = petunidin, Pe = peonidin, Mv = malvidin, gluc= glucose.

9.3 Results and Discussion

9.3.1 LC-UV Method Development

The method was developed with the following aims in mind: firstly, to be amenable to MS detection as identification was to be based on mass spectral data; secondly, sample preparation had to be kept to a minimum in order to eliminate loss of the labile anthocyanins and to ensure 100% recovery; finally, the method had to be robust enough to allow UV quantitation of the compounds identified by MS.

Method development was performed using filtered wine samples. In the first step, the mobile phase composition was optimised. The generic HPLC method for the analysis of anthocyanins is based on reversed phase LC with gradient elution employing acidified eluents. The low pH of the mobile phase is required to ensure that the anthocyanins are in the flavylium cationic form (~96% at pH 1.5), since the slow inter-conversion between the different chemical species at higher pH leads to severe peak broadening [26]. Also, under these acidic conditions, the anthocyanins absorb maximally at ~520 nm, leading to an improvement in sensitivity, and offering the opportunity of selective detection at this wavelength. Formic acid was chosen to adjust the pH because of its volatility and strong acid characteristics. The acid content of phase A (water) and phase B (acetonitrile) was evaluated between 1 and 10%. At 1% formic acid (pH of phase A is 2.1) broad peaks were observed. The peak height increased with acid content up to 7.5% (pH 1.6), where it remained relatively stable. This was chosen as the optimum acid content. It can be noted that even when working at this low pH, no loss of separation efficiency was observed after months of analyses using the same column. The gradient was tuned as specified in the Experimental section to deliver optimal separation of wine anthocyanins within an acceptable time.

Since the wine pigments are the only compounds absorbing in the region of 520 nm, this wavelength can be used for their selective detection and quantitation. This also means that, unless clear UV spectra are required for identification purposes, no sample preparation is

needed for the complex wine sample. As we made use of MS detection for identification, filtered wine samples were directly injected.

Due to a lack of available anthocyanin standards, external calibration was performed using malvidin-3-glucoside, and all compounds were quantified using this calibration graph. Linearity was checked over the range 5-250 $mg \cdot mL^{-1}$ (ppm) (triplicate injections at 5 levels, $R^2 = 0.9996$), and the limit of detection (S/N = 3) was determined as 0.184 ppm, more than sufficient for wine analysis.

9.3.2 LC-MS Identification of Wine Anthocyanins

Molecular ion and fragmentation information, together with λ_{max} values and relevant literature references for the identified compounds are presented in table 9.2. As expected, the predominant coloured species present in young red wines were those originating from the grape. Thus the 3-glucoside derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin (**1-5**, figure 9.1) were eluted in the specified order, with malvidin-3-glucoside being the major anthocyanins in all cases [26, 36-38]. λ_{max} values were lower for cyanidin-glucoside and peonidin-glucoside compared to the other 3, in agreement with [50]. Mass spectra contain the molecular ion $[M]^+$ as base peak, together with the fragment $[M-162]^+$, corresponding to the loss of the glucose moiety (figure 9.2) [41-44]. Cyanidin-glucoside was present in only trace amounts in most South African wines, but MS-MS experiments clearly showed the same spectra.

The corresponding 3-acetylglucoside- (**6-10**) and 3-p-coumaroylglucoside derivatives (**11-15**) of 5 specified anthocyanidins were identified in a similar way. The acetylglucoside-derivatives displayed, apart from the pseudo-molecular ion, another peak $[M-204]^+$, representing the aglycone after the loss of an acyl group. Coumaroylglucoside derivatives displayed the pseudo-molecular ion and the aglycone fragment at $[M-308]^+$ (loss of the p-coumaroyl-glucoside group, figure 9.2). The elution order for each anthocyanidin is glucoside < acetylglucosides < coumaroyl-glucoside [26, 36-38]. Coumaroyl-glucoside derivatives are additionally identified by a pronounced shoulder at ~310 nm in the UV spectrum, which is missing in both the other species [50]. In addition, malvidin-caffeoyl-

glucoside (**28**) was identified by its mass spectrum and retention time [42]. The molecular ion was detected at m/z 655, and the aglycone fragment at 331. Figure 9.1 presents the base peak chromatogram obtained for a South African red wine.

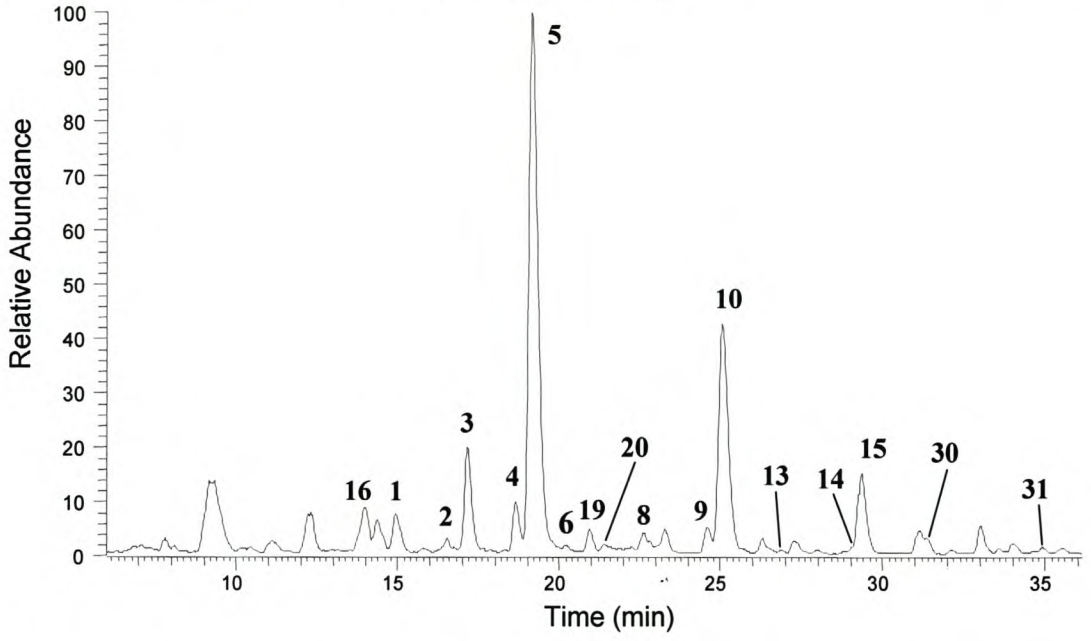


Figure 9.1: Base peak chromatogram obtained from the LC-MS analysis of a South African red wine. For method details, refer to Experimental. For peak identification, refer to table 9.2.

In addition to the grape anthocyanins, a number of derived pigments were identified in wine. A dimer resulting from the direct condensation of malvidin-glucoside and catechin/epicatechin (compound no. **16**) was detected at 14 minutes, displaying only the molecular ion at m/z 781. The occurrence of this compound in wine has been hypothesised by Somers [2], and more recently been proven [43]. The retention time is in agreement with that reported by Vivar-Quintana et *al.* [43].

No.	Rt	Compound	$[M+H]^+$	Fragments (MS)	Fragments (MS-MS)	λ_{max}	Reference(s)
1	15.0	delphinidin-3-glucoside	465	303	n.p.**	527	41-44
2	16.4	cyanidin-3-glucoside	449	287	449, 431, 287	517	41-44
3	17.1	petunidin-3-glucoside	479	317	n.p.	527	41-44
4	18.7	peonidin-3-glucoside	463	301	n.p.	516	41-44
5	19.2	malvidin-3-glucoside (mv-3-glc)	493	331	n.p.	527	41-44
6	20.2	delphinidin-(6-acetyl)-3-glucoside	507	303	n.p.	529	41-44
7	21.9	cyanidin-(6-acetyl)-3-glucoside	491	287	n.p.	519	41-44
8	22.6	petunidin-(6-acetyl)-3-glucoside	521	317	n.p.	529	41-44
9	24.6	peonidin-(6-acetyl)-3-glucoside	505	301	n.p.	516	41-44
10	25.1	malvidin-(6-acetyl)-3-glucoside	535	331	n.p.	529	41-44
11	26.6	delphinidin-(6-coumaroyl)-3-glucoside	611	303	n.p.	527	41-44
12	n.d.*	cyanidin-(6-coumaroyl)-3-glucoside	595	-	n.p.	-	41-44
13	26.9	petunidin-(6-coumaroyl)-3-glucoside	625	317	n.p.	536	41-44
14	29.1	peonidin-(6coumaroyl)-3-glucoside	609	301	n.p.	520	41-44
15	29.4	malvidin-(6-coumaroyl)-3-glucoside	639	331	n.p.	517	41-44
16	14.0	mv-3-glc-(epi)catechin	781	483, 331	n.p.	-	5-7,43
17	18.4	petunidin-3-glucoside-pyruvic acid	547	-	385	-	42-44
18	20.3	peonidin-3-glucoside-pyruvic acid	531	507, 303	463	-	42-44
19	21.0	Vitisin A (mv-3-glc-pyruvic acid)	561	399	n.p.	509	42-44
20	21.4	Visitin B (mv-3-glc-acetaldehyde)	517	355	n.p.	-	12,42-44
21	21.8	mv-3-glc-ethyl-(epi)catechin-unknown	1029	535, 331	-	-	42
22	22.7	mv-3-glc-ethyl-(epi)catechin	809	-	none	-	42-44
23	22.3	acetylvitisin A	603	399	n.p.	-	12
24	22.7	mv-3-glc-ethyl-(epi)catechin	809	-	none	-	42-44
25	23.1	acetylvisitin B	559	355	355	-	12,43
26	23.4	mv-3-glc-ethyl-(epi)catechin	809	-	none	-	42,44
27	25.7	coumaroylvitisin A	707	399	n.p.	-	44
28	26.0	malvidin-(6-caffeoyl)-3-glucoside	655	331	none	-	42
29	26.6	coumaroylvisitin B	663	355	none	-	43
30	31.3	Pigment A	609	447	n.p.	512	10,42,43
31	34.9	Pigment B	755	447	n.p.	-	10

Table 9.2: Retention times, mass spectral details and UV data of the anthocyanins identified in wine, together with cited references.

Pyranoanthocyanins resulting from reaction between anthocyanins and pyruvic acid were also detected. These adducts were detected only for those anthocyanins present in sufficient quantities: petunidin-glucoside (**17**), peonidin-glucoside (**18**) and malvidin-glucoside (**19**), and were identified by their MS spectra (containing a molecular ion 68 mass units greater than the respective glycosylated anthocyanins) and their retention times (eluting shortly after the respective anthocyanins) [42-44]. Also, λ_{max}–values were significantly lower than

for free anthocyanins (i.e. ~510 nm, compared to 527 nm) [11, 13]. The structure of compound **19**, named vitisin A by Bakker et *al.* [11] has been elucidated previously, although different structures were proposed [11, 13]. The loss of glucose from vitisin A (m/z 399, figure 9.2) was observed [12], while the same fragment was detected for petunidin-glucoside-pyruvic-acid (**17**, m/z 385) in MS-MS experiments. Pyruvic acid derivatives of malvidin-acetyl-glucoside (acetylvitisin A, **23**) and malvidin-coumaroyl-glucoside (coumaroylvitisin A, **27**) were also detected at m/z values of 603 and 707, respectively. A fragment at m/z 399 was detected for each of these compounds, resulting from loss of acyl- and coumaroyl-glucoside groups from **23** and **27**, respectively [12, 44].

An additional pyranoanthocyanin (**20**) resulting from the cyclo-addition of acetaldehyde to malvidin-3-glucoside, referred to vitisin B by Bakker et *al.* [12], was found at a retention time of 21.4 minutes. The mass spectrum showed, in addition to the molecular ion peak at 517, an aglycone fragment at m/z 355 (figure 9.2) [12, 44]. Similar products resulting from addition of acetaldehyde to malvidin-acetyl-glucoside (acetylvitisin B, **25**) and malvidin-coumaroyl-glucoside (coumaroylvitisin B, **29**) were also detected. The mass spectra showed, apart from the molecular ion (m/z 559 and 663 for **25** and **29**, respectively), the same aglycone fragment as observed for vitisin B at m/z 355 [12, 43].

Acetaldehyde-mediated condensation between malvidin-3-glucoside and (epi)catechin, leads to ethyl-bridged pigments [7, 8]. Three of the possible isomers of these pigments were elucidated (**22, 24** and **26**), with mass spectra (pseudo-molecular ion at m/z 809) and retention times in agreement with literature [42-44]. A related compound (**21**), detected at m/z 1029, corresponds to a possible product of polymerisation reactions involving these ethyl-bridged pigments [42].

New pigments formed by addition of 4-vinylphenol to malvidin-glucoside and malividin-coumaroyl-glucoside have recently been reported in wine media [9, 10]. Both compounds, referred to as pigment A and B [10] were found in this study. The mass spectrum of pigment A (**30**) showed a molecular ion peak at m/z 609, while the loss of glucose led to the fragment detected at m/z 447 (figure 9.2). The λ_{max} value for this compound is hypsochromically shifted to ~510 nm, compared to 527 nm for malvidin-glucoside, in accordance with [10]. Pigment B (**31**) displayed a similar mass spectrum, dominated by the

molecular ion at m/z 755 and the same aglycone fragment at 447. Representative mass spectra for a number of malvidin-derived pigments found in wine are shown in figure 9.2.

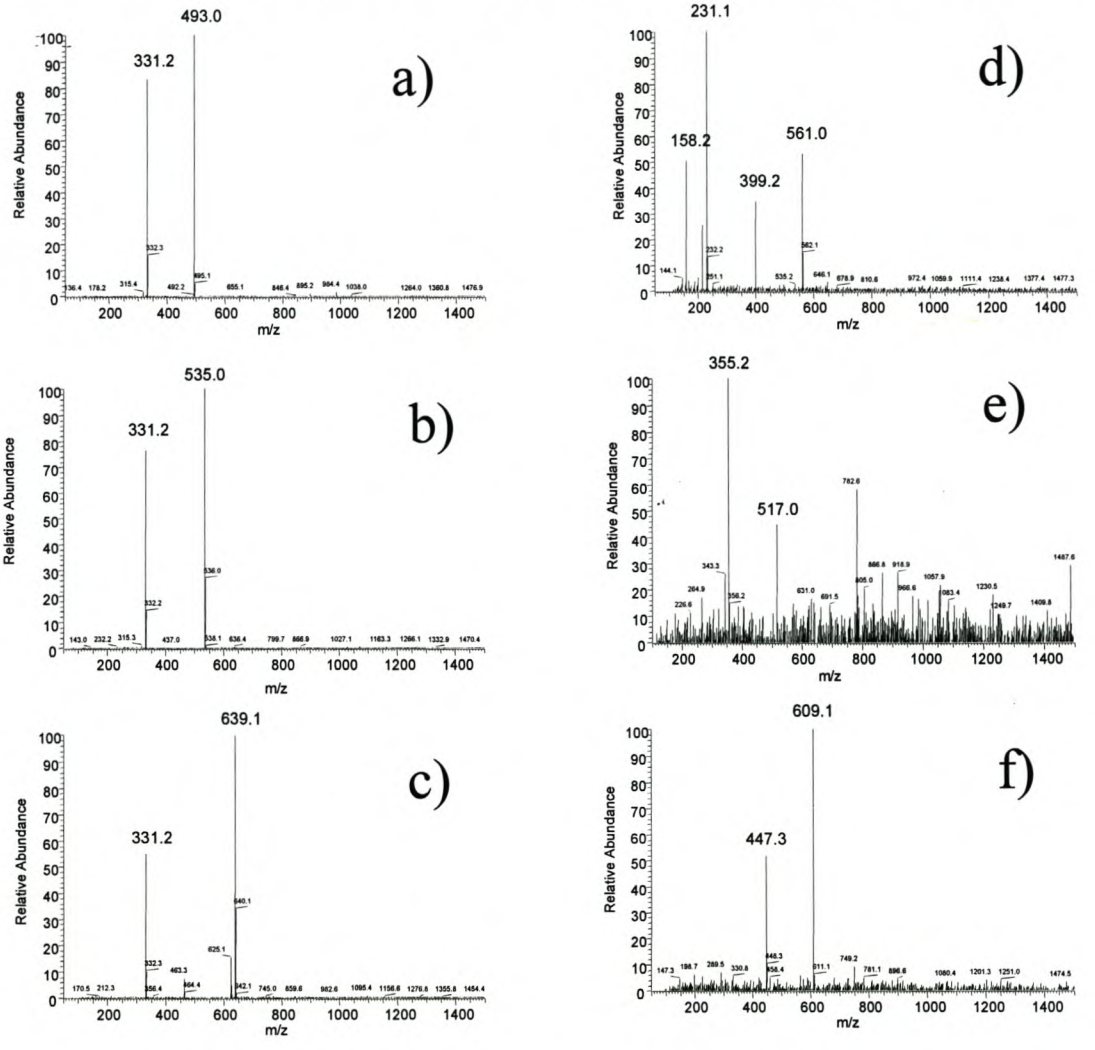


Figure 9.2: Positive electrospray spectra of some malvidin-derived pigments detected in red wine: a) malvidin-3-glucoside (5) b) malvidin-acetyl-glucoside (10) c) malvidin-coumaroyl-glucoside (15) d) vitisin A (19) e) vitisin B (20) and f) pigment A (30). For discussion of the spectra, refer to text.

9.3.3 Routine LC-UV analysis of wine anthocyanins

The power of LC-MS as identification tool for anthocyanins is evident from the precedent discussion. Chromatographic resolution of all the compounds listed in table 9.1 was not achieved, and in fact was not required in order to identify even those compounds present in trace amounts. However, for routine and quantitative analysis, UV detection is often preferred because of simplicity, reliability and lower cost. For this reason, 16 compounds were selected, based on their prevalence in most wines, to be quantified by UV detection in South African red wines. These compounds are specified in figure 9.3. The proposed procedure, together with chemometric methods of data analysis, will be used to obtain anthocyanin fingerprints of South African red wine cultivars. A qualitative comparison between UV chromatograms obtained for 5 South African red wine cultivars is presented in figure 9.3.

9.4 Conclusion

A robust HPLC-MS method was used to identify a total of 30 anthocyanins in wines, including grape pigments and products derived during ageing. Based on these results, an LC-UV method suitable for the routine analysis of 16 wine anthocyanins is proposed. Direct injection of filtered wine samples followed by selective detection at 520 nm allowed quantitation of these compounds in a wide variety of South African red wines. The LC method has the advantages of being rapid, reproducible and sensitive, making it the ideal tool for the characterisation of wines by their anthocyanin pattern.

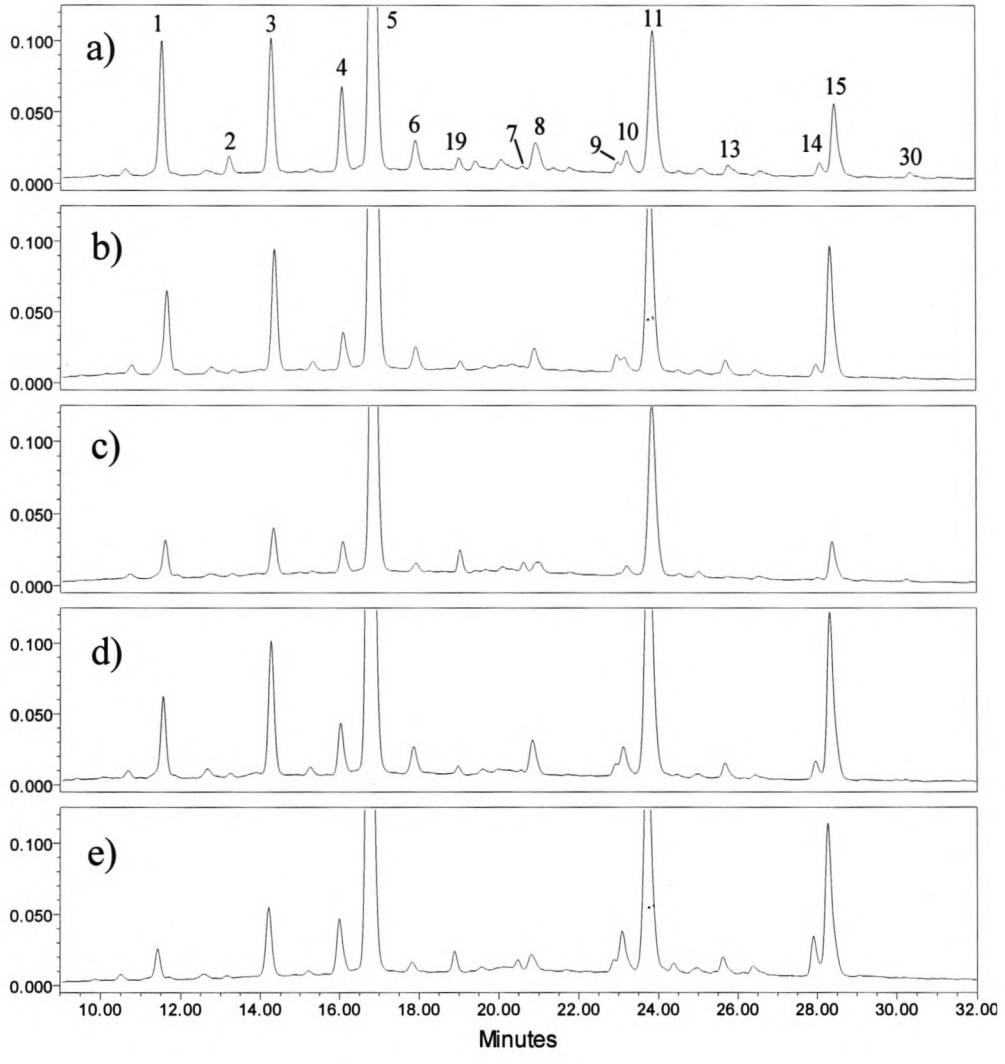


Figure 9.3: Comparison between UV chromatograms obtained for 5 South African red wines. The 16 anthocyanins chosen for quantitation purposes are indicated (for peak identification, refer to table 9.2). Wine cultivars: a) Merlot b) Ruby Cabernet c) Cabernet Sauvignon d) Pinotage and d) Shiraz.

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10

An Introduction to Chemometric Data Analysis

10.1 Introduction

The discipline of chemometrics has its origins in chemistry [1, 2], with applications ranging from simple evaluation of experimental data to the determination of structure-activity relationships. Analytical chemists in particular have made extensive use of chemometric methods in recent years for the purpose of obtaining relevant information from experimental data. This is largely due to the development of sophisticated, computerised analytical instrumentation that has led to the availability of a wealth of chemical information for a variety of samples. The often complex nature of the data obtained in this way has necessitated the use of statistical methods in order to efficiently extract the desired information.

Following Massart et *al.* [3], chemometrics can be defined as "a chemical discipline that uses mathematics, statistics and formal logic (a) to design or select optimal experimental procedures; (b) to provide maximum relevant chemical information by analysing chemical data; and (c) to obtain knowledge about chemical systems". Part (a) of this definition was not employed in this study, and will not be discussed further. The extraction of information from experimental data, part (b), may involve various (often related) manipulations, such as allow displaying, modelling or classification of the data [3]. Visualisation is needed in order to determine inherent grouping or structural relationships within the data, and for this purpose the analyst requires methods to display the data. In the special case of multivariate data, i.e. where multiple measurements are made on each sample, this becomes problematic since the number of dimensions needed to display the data is equal to the number of measurements made. A number of chemometric methods allow reduction of data dimensionality for displaying purposes (see below). Modelling involves the evaluation and description of relationships between variables or measurements such as used in univariate and multivariate regression. Classification methods deal with the problem of assigning objects to a certain class based on their measurement values. When the classes of objects are known (pre-defined), the classification process is referred to as supervised pattern recognition; otherwise unsupervised pattern recognition is used. For the chemist, the aim is finally to obtain a more meaningful interpretation of the data, aided by these methods, and

in this way to obtain more knowledge of the chemical system, the aim of part (c) of the definition. The chemometric procedures used in this work for the analysis of non-volatile compounds in wines will be outlined in the following section.

10.2 Univariate Statistics

When repeated analytical measurements of a specific property of a sample are made, the resultant data set commonly follows a normal distribution that is described by values for the mean and the standard deviation. The mean of a data set, $\bar{x}$, is given by:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \qquad (1)$$

where x_i is the ith measurement and n is the total number of measurements made. The standard deviation, s, can be calculated from:

$$s = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \qquad (2)$$

The variance is defined by the square of the standard deviation, s^2, while the relative standard deviation, expressed as a percentage, is given by :

$$\mathrm{rsd}(\%) = 100 \times (s / \bar{x}) \qquad (3)$$

The comparison of variances from two measurement sets, s_1^2 and s_2^2, is performed by an F-test, defined by:

$$F = \frac{s_1^2}{s_2^2} \qquad (4)$$

where s_1^2 is the larger of the two variances. This F-value (F_{calc}) is then compared to a critical F-value, F_{crit}, obtained from an F-distribution as defined by a chosen significance level (normally 95% or 99%) and the degrees of freedom corresponding to s_1^2 and s_2^2 (= n_1

- 1 and n_2 - 1, respectively). The two variances are considered significantly different if the calculated value is larger than F_{crit}.

In order to determine whether two or more sets of measurements differ significantly or only at random, analysis of variance (ANOVA) is performed. In this procedure, the total variance is partitioned into variances within different groups (i.e. sets of measurements) and variances between groups. These variances can then be compared using an F-test, defined by the variance due to the group effect divided by the residual variance. For a calculated F-value higher than F_{crit}, the measurements are said to differ significantly. The *effect* the additional variance added by the measurement groups is thus determined to be significant [4]. This is an example of one-way ANOVA. Two-way or multi-way ANOVA is used when two or more effects on the data have to be determined.

10.3 Multivariate Statistics

When a number of measurements are obtained for a set of samples (for example a number of chemical constituents are quantified in various samples), the resultant multidimensional data can be arranged as a data matrix $\underset{\mathbf{n\times p}}{\mathbf{X}}$, where by convention the samples are arranged in rows, and the measurements are the columns (the subscripts n and p indicate the number of columns and rows, respectively):

$$\underset{\mathbf{n\times p}}{\mathbf{X}} = \begin{pmatrix} x_{11} & \cdots & x_{1p} \\ \vdots & \ddots & \vdots \\ x_{n1} & \cdots & x_{np} \end{pmatrix} \qquad \textbf{(5)}$$

where x_{ij} is the value of the jth measurement performed on the ith sample. Samples are commonly referred to as objects and measurements as variables. Related objects (objects with similar values for the variables) are said to belong to the same class. A specific sample i can be represented by the vector $x_i = (x_{i1}, x_{i1} \ldots x_{ip})$ in the p-dimensional space defined by the variables. When $p \leq 3$, the vectors can be plotted to determine similarity between objects.

In cases where it is necessary to correct for the differences in measurement units and variances between variables, auto-scaling is performed [5]. This process provides variables with zero mean and unit variance, according to:

$$z_{ij} = \frac{x_{ij} - \bar{x}_j}{s} \tag{6}$$

The covariance of two variables is a measure of the extent to which these variables follow similar trends (i.e. vary together). Variance-covariance (or simply covariance) and correlation matrices demonstrate this relationship among the variables, and are used extensively in factorial methods. The covariance matrix is calculated using the variance, s^2 (from eq. **(2)**), and the covariance, defined by:

$$\mathrm{cov}(i,j) = \frac{\sum_{i=1}^{n}(x_{ij} - \bar{x}_j)(x_{ik} - \bar{x}_k)}{n-1} \tag{7}$$

The covariance matrix has the form:

$$\mathbf{C} = \begin{pmatrix} s^2_{11} & \mathrm{cov}(1,2)\cdots & \mathrm{cov}(1,p) \\ \vdots & \ddots & \vdots \\ \mathrm{cov}(p,1) & \mathrm{cov}(p,2)\cdots & s^2_{pp} \end{pmatrix} \tag{8}$$

The correlation matrix can be derived from the covariance matrix by determining the correlation coefficients, r_{jk}, using:

$$r_{jk} = \frac{\mathrm{cov}(j,k)}{s_j s_k} \tag{9}$$

In the case of autoscaled data, the covariance matrix is equal to the correlation matrix.

10.4 Multivariate Data Analysis

The extraction of meaningful information from multivariate data can be performed in a number of ways. Pattern recognition methods allow the analyst to investigate the structuring of the data. In the first instance exploratory data analysis can be performed using the methods of factor analysis [6]. The particular aim of these methods is to reduce the dimensionality of the data by extraction of common factors. In factor analysis interpretable factors are extracted, to which a physical meaning can be attached [7]. Principal component analysis is the most popular form of factor analysis where the extraction of principal components occurs on the basis of the maximum variance explained. Cluster analysis is a second unsupervised pattern recognition method used to classify objects into groups based on their description by a set of variables. In supervised pattern recognition a classification rule is derived from a set of training objects belonging to a known class, and this is used to classify new objects. Discriminant analysis is a commonly used example of a supervised pattern recognition technique.

10.4.1 Unsupervised Pattern Recognition

10.4.1.1 Principal Component Analysis

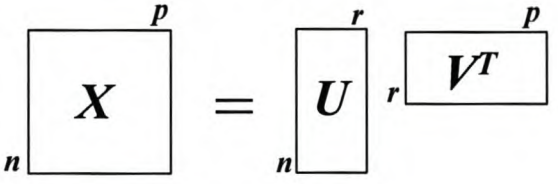
The basis of principal component analysis (PCA) is the transformation of a set of correlated variables to a set of uncorrelated factors (called principal components or PC's) on the basis of the maximum variance criterion. The PC's, also referred to as latent variables, are in fact linear combinations of the original variables, or manifest variables. The usefulness of PCA stems from the fact that the latent variables are better descriptors of the data structure than the manifest variables [8]. Since a large part of the variance of the data set can be explained by a limited number of latent variables, PCA leads effectively to the reduction of the dimensionality of the data. This also makes it possible to plot the first few PC's in order to examine the data for possible groupings or patterns, detect possible outliers or tentatively

classify samples. From the manner in which the latent variables are extracted, superfluous manifest variables can be identified, thereby allowing data reduction [9].

In principle, PCA involves the generation, starting from p manifest variables, of p latent variables or PC's, each being a linear combination of the original variables. This is achieved by decomposition of the data matrix $\underset{n\times p}{\mathbf{X}}$ (see **(5)**) to two matrices, according to:

$$\underset{n\times p}{\mathbf{X}} = \underset{n\times r}{\mathbf{U}}\underset{r\times p}{\mathbf{V}^T} \qquad \textbf{(10)}$$

or, schematically:



(Here ^{T} designates the transpose of the matrix, obtained by interchanging the rows and columns, and $r \leq p$). V is a matrix containing as columns the eigenvectors for the latent variables, and as rows the projections of the original variables on these latent variables (referred to as the loadings). This matrix thus describes the relation between the manifest and latent variables, and is known as the loading matrix.

U is referred to as the scores matrix, containing as columns the projections of the n objects on the latent variables (called scores). This matrix describes object positions in the r-dimensional PC space. The elements of the scores matrix (the scores) can be determined by rewriting eq. **(10)** as:

$$\mathbf{U} = \mathbf{XV} \qquad \textbf{(11)}$$

Taking PC1 as an example, the scores of the objects on this PC can be obtained by matrix multiplication follows:

$$u_{11} = x_{11}v_{11} + x_{12}v_{21} + \cdots + x_{1p}v_{p1} \qquad \textbf{(12)}$$

$$u_{21} = x_{21}v_{11} + x_{22}v_{21} + \cdots + x_{2p}v_{p1}$$

$$\vdots$$

$$u_{n1} = x_{n1}vz_{11} + x_{n2}v_{21} + \cdots + x_{np}v_{p1}$$

where it is clear that the new scores are linear combinations of the manifest variables.

An inherent feature of the latent variable extraction process is that the obtained factors are uncorrelated; PCA can be said to decorrelate variables [10]. Also, the scores for each subsequent latent variable are chosen so as to maximise the variance within that PC (i.e. large differences in the scores of the objects on this PC), subject to the restriction that the scores between latent variables be uncorrelated. Generally, the scores of PCi are chosen to make the variance within this PC as large as possible, while at the same time being uncorrelated with PC1 to PC(i-1) [11].

Often the last few latent variables account for a negligible amount of the variance (they essentially express noise), and these PC's can be ignored. The choice of significant latent variables to be used can be made based on several criteria. Thus the percentage of explained variance can be calculated and is often used to select those PC's that cumulatively explain say 80% of the variance. According to the eigenvalue-one criterion, only PC's with eigenvalues greater than one are selected (the mathematical basis of this criterion will not be explained here).

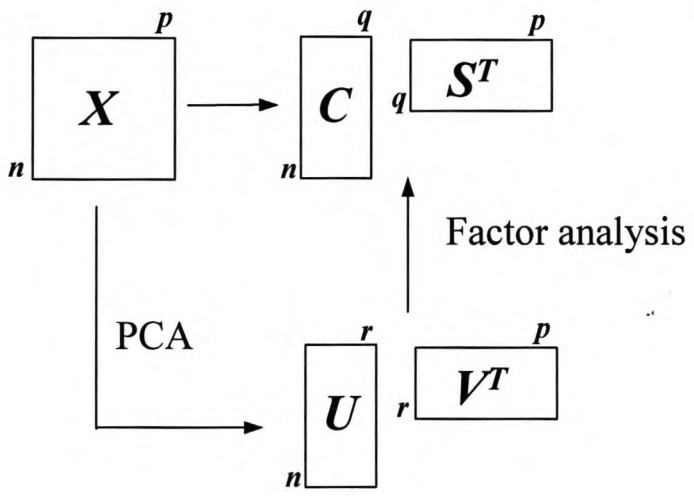
The results of PCA are usually interpreted visually. Thus either the scores of objects or the loadings of manifest variables can be plotted against selected latent variables, to obtain scores- and loadings plots, respectively. After suitable scaling, the loadings can be superimposed on a score plot to produce a so-called bi-plot, allowing concurrent interpretation of scores and loadings.

One of the main aims of PCA is pattern recognition. The score plot is a valuable tool for examining the data (objects) in the space defined by the latent variables. Relationships between objects and their possible grouping can be determined. From the loadings plot, the correlation between variables can be determined. The angle between loading vectors is indicative of their correlation, with small angles implying high correlation (orthogonal vectors are uncorrelated). For highly correlated variables, only one variable can be used in subsequent analysis, since these variables account for a similar amount of variance. In this manner PCA allows manifest variable reduction. The size of the loading (i.e. the projection of a loading vector on a specific latent variable) is a measure of the contribution of this variable to the variance explained by this PC. From this the discriminating power of the variable can be deduced. For example, variables with high positive loadings on PC1 are

important in differentiating between objects with high scores on this PC from those with low or negative scores [12].

10.4.1.2 Factor Analysis

The latent variables extracted in PCA (the PC's) are said to be abstract factors. Because PCA is performed under the constraints mentioned above (maximum variance criterion and orthogonality), these abstract factors rarely have any interpretable physical significance. In contrast, in factor analysis (FA) the aim is to obtain factors with physical meaning, for example a factor can be related to the concentration of a non-measured variable. The extraction of these so-called true factors is normally performed by transformation of abstract factors, obtained for example by PCA. This transformation is performed by rotation of the PC's in such a way that interpretable factors are obtained. Varimax rotation is commonly used for this purpose. Here rotation of a PCA loading matrix is performed using an orthogonal rotation matrix. This rotation is performed in such a manner as to produce maximal row simplicity, since true factors should be easily interpretable [13]. A schematic representation of the relation between PCA and FA is presented below:



10.4.1.3 Cluster analysis

Cluster analysis (CA) is an alternative unsupervised pattern recognition technique used to group objects based on their variable composition. Objects are grouped based on their similarity, and the assumption is that this can be measured by the distance between objects in the space defined by the variables. Various measures of the distance between objects can be used. The Minkowski distance provides a general measure of the distance between objects *i* and *j* according to [12, 14]:

$$d_{ij} = \left[\sum_{k=1}^{K} \left| x_{ik} - x_{jk} \right|^{p} \right]^{1/p} \qquad (13)$$

where K is the number of variables. For $p = 2$, the Euclidean distance is calculated. In this study, the Manhattan distance (also called city-block distance) was used, this is defined as above by $p = 1$. The Manhattan distance is the sum of the distances parallel to the axes. For cases where the ranges (difference between the maximum and minimum values of a variable) of variables are unequal, range scaling is performed prior to CA.

The most common clustering method is agglomerative hierarchical clustering, where objects are combined according to their distance from each other starting with single objects and merging them to larger clusters. Objects can be aggregated by use of various methods. Ward's method entails aggregation in a manner that leads to a minimum increase in heterogeneity of all clusters (heterogeneity is defined as the sum of squared distances of objects to the centroid of clusters), and was used in this study [14].

Graphically, the results of CA are depicted in a dendrogram, where the linkages between objects are plotted against the distance measurement. The number of clusters (groups of similar objects) if unknown, can be selected based on a pre-determined distance measure between clusters.

10.4.2 Supervised Pattern Recognition

In supervised pattern recognition, a set of objects of known classification (i.e. belonging to a known class) is used to derive a classification rule, whereby samples of unknown class

can consequently be categorised into the known objects groups. Various pattern recognition methods can be used for this purpose; essentially these methods differ in the way in which the classification rules are defined. Primarily two approaches are encountered, depending on whether the emphasis is on the discrimination between classes or the modelling of classes [15]. Linear discriminant analysis, as one of the best-known supervised pattern recognition techniques, is an example of the former techniques, which will be briefly discussed below.

10.4.2.1 Linear Discriminant Analysis

The first step in linear discriminant analysis (LDA) is the selection of a suitable training set consisting of a number of samples or objects of known classification, described by a certain number of variables. Next, variables suitable for the differentiation between classes of objects are selected. In the special case of forward stepwise discriminant analysis, manifest variables are evaluated for their contribution to the discrimination between classes by their respective *F*-values [16]. Variables are added to the model in order of decreasing discriminating power. Following variable selection, a classification rule is derived, as described below.

Analogues to PCA, linear discriminant analysis (LDA) can be considered as a variable reduction technique. However, the criterion for selection of latent variables in LDA is that the projection of the objects onto these variables should achieve the maximum differentiation between the known classes (where in PCA the projection retains maximal variance). Mathematically, this means that the ratio of between-class variance to within-class variance is maximised. The latent variables obtained in this manner are also linear combinations of the manifest variables, and are called canonical variates (for n classes, n-1 canonical variates can be determined). The projection of a manifest variable on each latent variable is a measure of the specific variables' contribution to the discrimination allowed by this latent variable. For each class the location of the point that represents the means for all variables in the multivariate space defined by the latent variables can be determined; this point is known as the group (or class) centroid. An object is then classified as belonging to that class to which the distance to the centroid is a minimum. In this case, the Mahalanobis

distance was employed, since this measurement accounts for the different scales and variances between variables, as well as their correlation [12]. The Mahalanobis distance between objects *i* and *j* is calculated using:

$$D_{ij}^2 = \left(\mathbf{x}_i - \mathbf{x}_j\right)^T \mathbf{C}^{-1} \left(\mathbf{x}_i - \mathbf{x}_j\right) \qquad \textbf{(14)}$$

where $\mathbf{C}$ is the covariance matrix, and $\mathbf{x_i}$ and $\mathbf{x_j}$ are the column vectors for objects *i* and *j*, respectively.

The final step in LDA is the validation of the derived classification rule. The recognition ability of the model is determined as the percentage of objects in the training set that are correctly classified. In contrast, the prediction ability is evaluated by classifying objects of known class, termed the test set (ideally, a separate set of objects from those used for the training set are included in the training set). The prediction ability is thus characterised as the percentage of objects in the test set that are correctly classified [15].

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11

Chemometric Investigation of the Non-Volatile Composition of South African Wines

11.1 Introduction

Knowledge of the chemical content of wine is of great importance throughout the field of oenology, and this for various reasons. Such knowledge is primarily used to enhance winemaking practice with the result of the production of more consistently fine wines. In recognition of this fact, a very large body of data regarding the chemical constituents of wine has been built up, especially during the last two decades. However, meaningful interpretation of such vast amounts of data, such as to allow the relation of chemical composition to certain wine properties (most importantly, sensory properties), is often problematic. Statistical, and in particular multivariate, methods offer the possibility of fast and efficient information extraction from large sets of data. Since the first reports on the application of multivariate statistical methods in oenology [1, 2], the number of papers dealing with this topic has increased rapidly. Although these so-called chemometric methods are nowadays used to study various processes during winemaking [3-5], one of the principal applications has been the differentiation and classification of wine samples, based on their chemical composition, according to some property such as geographical origin, variety (cultivar) or quality. In combination with chemometric methods, diverse chemical data have been employed in order to achieve these goals.

In this regard, the metallic content of wines have often been used to differentiate wines according to their geographical origin [6-8]. Isotopic determinations in conjunction with metals have also been used for this purpose [9]. Metal content has been reported to vary slightly according to the type of wine [7] and the vintage [6], although classification was not performed on this basis. The content of inorganic ions in conjunction with volatile data and classical wine parameters proved successful to classify white wines according to geographical origin, independent of the vintage [10]. Similarly, blended Spanish red wines of two vintages were classified according to their origin based on their metallic and phenolic content [11].

Other authors have used amino acid profiles to differentiate wines according to variety, vintage and geographical origin [12, 13], although varietal differences seem more pronounced. Together with volatile and metal composition, the amino acid content has been

used to classify Italian wines of the same variety according to their vintage [14]. Biogenic amines together with amino acid [13] and phenolic data [15] have been used to differentiate Hungarian red and white wines.

Moreover, chemometrics have been used together with sensory data, for example to classify wines by their organoleptic profile [16]. The relationship between chemical measurements (metals, volatiles) and sensory evaluation for Pinot Noir wines from France and America was studied using partial least squares (PLS, a multivariate regression method) [17]. Chemical data proved more useful than sensory data for the classification according to geographical origin. A predictive model was calculated that made possible the prediction of certain sensory characteristics from chemical variables (especially metallic content), although it is doubtful whether these results can be extended to other wines. Nogueira and Nascimento [18] noted poor discrimination of Madeira wines when using sensory descriptors, while good differentiation of these wines according to type (dry, medium dry, medium sweet, sweet) and vintage was obtained for physicochemical data (classical determinations and volatiles). In a study on the classification of French wines from 4 regions, slightly better results were also obtained from chemical data (classical measurements, organic acids, volatiles) compared to sensory data [19]. Classical parameters and volatile composition were also employed by Aleixandre et *al.* to differentiate 4 Spanish wine varieties, independent of geographical origin, wine-making practice and vintage [20].

The phenolic compounds constitute another class of compounds that have proved useful for the differentiation of wines using chemometric methods. Thus, polyphenol data have been shown to be more useful than volatile, amino acid and classical data to differentiate Spanish wines according to grape variety [21]. Particularly the concentrations of the tartaric acid ester of p-coumaric acid and caffeic acid proved important in this case, since the production of these compounds in grapes are thought to be under genetic control [22]. On the other hand, volatile data together with total phenol and flavonoid amounts was used to differentiate these wines according to vintage, while amino acid content together with volatile data allowed differentiation between wines from several wineries. Polyphenols present in white, blanc de noir and rosé wines from Spain allowed their differentiation and classification [23]. Moreover, polyphenols have successfully been used to classify red [24]

and rosé [25] wines from Spain according to their geographical origin, in the latter case independent of vintage.

In red wines, the anthocyanins have received a lot of attention as important compounds in the differentiation between cultivars. Anthocyanins in wine originate from the skins of black grapes, from where they are extracted during maceration. The relative amounts of glucosides, acetyl-glucosides and coumaroyl-glucosides have been suggested to differentiate between grapes [26]. Although the accumulation of anthocyanins shows variations during grape ripening, the anthocyanin fingerprints remain characteristic for each variety [27]. However, there is evidence that this pattern is affected by the climate during ripening, and as a result does vary within a grape variety between vintages [27]. The anthocyanin profile of grapes has been shown to differ from that of the wine made from them, a result ascribed to differing extraction efficiencies for these compounds, as well as degradation and condensation reactions that take place during winemaking. Once again, though, the wine anthocyanin profile remains characteristic for each variety [28, 29]. It has also been shown that the relative amounts of wine anthocyanins remain constant during wine ageing during 8 months in the barrel [29]. All these data suggest that the anthocyanin fingerprint of wines can be used to effectively characterize them by grape variety. Indeed, differentiation of wines by variety, as well as geographical origin, by anthocyanin content has been reported [30]. In this study, selected anthocyanins and polyphenols were used to classify 6 French red wines according to variety, independent of geographical origin. Further, differentiation of German wines [31] and classification of Spanish wines [32] according to cultivar has been demonstrated using anthocyanin fingerprints. In the latter study, malvidin-acetyl-glucoside was influential in differentiating the wines according to variety. It was also noted that the levels of p-coumaroyl-glucoside allowed differentiation of these wines according to origin [32]. In agreement with these findings, the ratio of non-acetylated- to acetylated anthocyanins was found to be influential in differentiating wines by geographical region [33]. In another study, the aim of which was to classify Greek wines by their geographical origin, the anthocyanin content proved to be more useful for this purpose than metallic, phenolic and sensory data [34]. Related chromatic parameters have also been use for the multivariate characterization of wines according to ageing status [35] and origin [36].

A number of multivariate statistical methods have been shown to be effective in the studies mentioned above. Pattern recognition methods are particularly useful as tools for investigating the structuring of the data. Thus, exploratory data analysis is usually performed using the methods of factor analysis, and the most frequently used method is that of principal component analysis (PCA) [7-8, 18-20, 34]. PCA reduces the dimensionality of the data by extraction of latent variables (PC's) on the basis of the maximum variance explained, thereby allowing the plotting and visualisation of the data using a reduced number of PC's. PCA is an unsupervised pattern recognition method, since the groupings of the data are not pre-determined. Cluster analysis (CA) is another unsupervised pattern recognition method commonly used for investigating groupings within wine data [7, 18]. In CA, objects (wine samples) are classified into groups based on their similarity, assumed to be related to the distance between these objects in an n-dimensional space defined by the n variables (measurements). In the same way, variables can be grouped according to their values in p objects.

Supervised pattern recognition methods, on the other hand, are used for classification of wines according to some criterion. Here, a classification rule is derived from a set of wines of known class, and this is used to classify unknown wines. The efficacy of discriminant analysis (DA) for the classification of wines has been demonstrated by numerous authors [7-8, 18-20, 24-25]. For a more complete overview of each of these methods, we refer to Chapter 10.

From the above discussion it is evident that the use of chemical data together with chemometric methods is an important tool for the classification of wines. The classification according to variety, vintage and geographical origin is especially important from an authenticity point of view [37]. However, all these factors simultaneously affect the chemical composition of wine, and thus the possible characterization of wines in this manner. For example, variations in chemical content between vintages and wine-making regions (and practices) obscure the differentiation according to grape variety, and vice versa. Notwithstanding this fact, there is sufficient evidence that by careful evaluation of the data and use of statistical methods, it is possible to effectively characterize wines by their chemical content according to a specified criterion. The aim of this study was to obtain a classification model, by chemometric methods, to allow the classification of South

African red and white wines according to variety, independent of the effects of vintage and geographical origin, based on their non-volatile composition.

11.2 Experimental

11.2.1 Samples

Sixty-two red and thirty-eight white wines of various vintages ranging from 1988 to 2003 were either purchased commercially, or were kind gifts from the KWV and the South African National Wine Show Association. Red wines included the varieties Cabernet Sauvignon, Merlot, Shiraz (Syrah), Pinotage, Ruby Cabernet and Tinta Barroca, as well as a number of blends. White wines were of the varieties Chardonnay, Sauvignon Blanc or Chenin Blanc. The wines used in this study are listed according to variety in table 11.1. Details of the wines analysed can be found in Addendum A. When not analysed from freshly opened bottled, wine samples were transferred under nitrogen to completely filled amber bottles to ensure their preservation.

Red wine cultivars	No. samples	White wine cultivars	No. Samples
Cabernet Sauvignon	13	Chardonnay	15
Merlot	10	Chenin Blanc	10
Pinotage	11	Sauvignon Blanc	13
Ruby Cabernet	10		
Shiraz	11		
Tinta Barroca	1		
Blends	6		

Table 11.1: The wine samples analysed in this study.

11.2.2 Analytical Methods

A total of 22 non-coloured phenolic compounds were quantified in the red and white wines by RP-LC-DAD. Filtered white wines were directly injected (20 μL), while sample clean-

up by SPE was performed for red wines. Details of the method can be found elsewhere [38]. Compounds were identified by comparison of their retention times and UV spectra with available standards, and quantified using calibration curves constructed for the standards. Phenolics for which the standards were unavailable were identified by LC-MS, as described in Chapter 8, and quantified using calibration curves of related standards. Thus, caffeoyl-tartaric acid (cafta) was quantified as caffeic acid, coumaroyl-tartaric acid (couta) as p-coumaric acid, myricetin-glucoside, quercetin-glucoside, isorhamnetin-glucoside and kaempferol-glucoside as rutin, trans-polydatin as trans-resveratrol, cis-polydatin as cis-resveratrol and isorhamnetin as kaempferol. Cis-resveratrol was obtained from a solution of trans-resveratrol by irradiation using a UV lamp (254 nm, Mineralight Lamp, Ultra-violet Products, CA, USA) for 30 minutes. The amount of cis-resveratrol formed was determined by quantifying the loss of trans-resveratrol, as no other reaction products were observed [39, 40], and subsequently used for calibration purposes.

Fourteen free anthocyanins and two derived products were quantified by direct injection-RP-LC-DAD analysis, as described in Chapter 9. Identification of these compounds was based on LC-MS and UV data. All sixteen compounds were quantified as malvidin-3-glucoside (Extrasynthese, France) equivalents.

Fructose and glucose were quantified by direct injection-NP-LC-RI following the procedure described in Chapter 6.

The six principal organic acids in wines (tartaric, malic, lactic, acetic, succinic and citric acids) were determined using CE with indirect UV detection, as described in [41].

The pH of wine samples was measured using an Orion Model 420A pH-meter (Labotec, South Africa). All results are given as mean values of duplicate determinations.

Table 11.2 lists the forty-seven chemical compounds analysed together with their abbreviations, as used in this chapter.

Anthocyanins	Abbrev.
cyanidin-3-glucoside	Cg
cyanidin-(6-acetyl)-3-glucoside	CgAc
delphinidin-3-glucoside	Dg
delphinidin-(6-acetyl)-3-glucoside	DgAc
delphinidin-(6-coumaroyl)-3-glucoside	DgCm
malvidin-3-glucoside	Mg
malvidin-(6-acetyl)-3-glucoside	MgAc
malvidin-(6-coumaroyl)-3-glucoside	MgCm
malvidin-3-glucoside-4-vinylphenol (A)	MgVp
Vitisin A (mv-3-glucoside-pyruvic acid)	MgPA
peonidin-3-glucoside	Png
peonidin-(6-acetyl)-3-glucoside	PngAc
peonidin-(6coumaroyl)-3-glucoside	PngCm
petunidin-(6-acetyl)-3-glucoside	PtgAc
petunidin-(6-coumaroyl)-3-glucoside	PtgCm
petunidin-3-glucoside	Ptg
Polyphenols	**Abbrev.**
caffeic acid	CA
caffeoyl-tartaric acid	CTA
catechin	C
epicatechin	EC
protocatechuic acid	PrCA
p-coumaric acid	PCA
ferulic acid	FA
gallic acid	GA
kaempferol	Ka
myricetin	My
myricetin-glucoside	Mg
cis-polydatin	CPd
trans-polydatin	TPd
quercetin	Q
quercetin-glucoside	Qg
resveratrol	Re
cis-resveratrol	CR
isorham/Kaempf-glucoside	IrKg
isorhamnetin	Ir
syringic acid	SA
coumaroyl-tartaric acid	CMA
vanillic acid	VA
Organic acids	**Abbrev.**
tartaric acid	TA
succinic acid	SAc
acetic acid	AA
lactic acid	LA
citric acid	Cac
malic acid	MA
Sugars	**Abbrev.**
fructose	F
glucose	G

Table 11.2: Chemical compounds quantified in South African wines, together with their abbreviations.

11.2.3 Statistical Methods

Two data matrices were constructed from the analytical data obtained for red and white wines, respectively, with rows represented by wine samples (objects) and columns corresponding to chemical measurements (variables). Autoscaling was performed to produce variables with zero means and unit standard deviation [42]. To start off with, the complete data matrices for red and white wines were split in three, each containing the data of the anthocyanins (red wines only), polyphenols, and acids plus sugars and pH on the entire set of wines. This was done to avoid masking effects of different classes of compounds on the classification. Each of the five matrices was investigated by statistical

methods to determine which compounds in each class were important in the differentiation between wine varieties.

Initially, univariate characterization was carried out based on Fischer's weight (F) by means of one-way ANOVA to establish which compounds differ significantly between varieties. PCA was performed in order to investigate the variance within each data set as described by each variable, and to check for correlation between variables. Varimax rotation was performed in each case to obtain maximal information from the extracted PC's [43]. CA was used as complementary unsupervised pattern recognition technique to investigate the grouping between measurements. Linear discriminant analysis (LDA) was used to derive a classification rule whereby the wine samples were classified according to variety. Both standard and stepwise forward LDA were performed, and the results are discussed for the model which produced the best classification results. Blended red wines were not included in this classification, for obvious reasons, and neither was Tinta Barroca, since only one wine of this variety was analysed.

Subsequently, the complete data set (containing red and white wines, but excluding the anthocyanin data) was used to classify wines according to cultivar. An example of the classification of an unknown (test) sample is then described, where a random sample is left out and LDA is performed.

All statistical data analysis was performed using STATISTICA, version 6.1 (Statsoft Inc., OK, USA).

11.3 Results

11.3.1 Comparison of Results With Literature Data

Initially, a brief comparison with published data for the compounds analysed in this study was made to investigate similarities or differences in chemical content of South African wines as compared to wines from other countries. The analytical results for all the wines can be consulted in Appendix B. In the following discussion, only the mean values for each variety will be mentioned.

11.3.1.1 Red Wines

The mean values for catechin and epicatechin in the different cultivars (32-58 ppm for catechin, 17-37 ppm for epicatechin) compare favourably with those reported for South African wines in a comprehensive study by Goldberg et *al.* [44], with the exception of Shiraz, where lower values were reported for both compounds. The levels were on the low side compared to wines from all over the world, although similar to those reported for Australian and Italian wines. Also in agreement with this study, Merlot wines contained on average higher amounts of these compounds compared to the other varieties (although significantly less than Pinot Noir wines, not analysed here). Values obtained in the current study are also similar to those reported in a second study of Canadian wines, according to cultivar, by Soleas et *al.* [45], and those reported for French wines of the same varieties as studied here [46]. p-coumaric acid levels determined in the current study (mean values for the cultivars ranging from 5.7-8.3 ppm) were higher than those reported for South African wines (especially in the case of Pinotage, which had the highest levels), and significantly higher than values in the same cultivars from various countries [47], and Canada [45]. Quercetin concentrations were almost twice as high in the cultivars Merlot and Shiraz (~15 ppm), compared to Cabernet Sauvignon and Ruby Cabernet, with Pinotage displaying the lowest mean concentrations (4.9 ppm). A similar pattern was reported for wines from all over the world [47]. Also in agreement with this study, quercetin levels in South African wines are higher than those found in any other country (levels in Australian wines are only slightly lower). For two other flavonols, kaempferol and myricetin, levels found in this study exceed those reported in Spanish wine [48]. Mean values for vanillic acid (3.6-5.8 ppm) and ferulic acid (0.4-0.6 ppm) are higher and lower, respectively, than those reported for the same Canadian cultivars [45]. Mean values for gallic acid are similar to those reported for French wines [46], and gallic- and protocatechiuc acid levels are higher than those found in Spanish wines [48]. For caffeic acid, mean values similar to those found in French [46] and Canadian [45] wines of the same cultivars, were obtained (7.7-17.4 ppm), while Pinotage was characterised by significantly higher levels (mean 33 ppm, although the content of this compound varied widely within this cultivar). The tartaric acid ester of this

compound, cafta, showed a similar trend: significantly higher values in Pinotage, and similar values in other varieties as reported [45], with large variations within a cultivar. In fact, a clear inverse relationship between caffeic acid and cafta concentrations was evident in Pinotage (wines containing high amounts of the former contained less of the latter), although the differences between cultivars for these compounds as reported above were maintained. Concentrations of the tartaric acid ester of p-coumaric acid, couta, were highest in Pinotage and Shiraz, and higher than values reported previously [49]. No clear trend was observed between the amounts of p-coumaric acid and its tartaric acid ester, as observed for caffeic acid. Of particular interest, are the levels of the various stilbenes-derivatives found in South African wines, in comparison with the vast amount of data reported for these compounds in wines from all over the world in recent years. In a global survey (excluding South Africa) of trans-resveratrol content in red wines, Goldberg et *al.* [50] reported mean values ranging between 1.5 and 4.4 ppm, which are higher than those determined in this study (means varied between 0.7 and 2.1 ppm for the cultivars, with Merlot concentrations being the highest). Compared to [50], other reports revealed similar [51-53], or slightly lower [45, 54,55] concentration ranges for this compound. Cis-resveratrol concentrations reported here (mean values 0.4-1.6 ppm, for Cabernet Sauvignon and Merlot, respectively) are similar to those reported for Portuguese wines [53, 54]. Other authors reported slightly lower values [45, 52], while studies dealing with wines from various origins were also in general agreement with our values [51, 55]. For the polydatins (mean concentrations for trans- and cis-polydatin varying between 0.3-3.0 and 0.2-2.9 ppm, respectively), similar trends were detected for South African wines: the values are in general agreement, if on the lower side, of the ranges reported in the literature [53, 55].

Comparison of anthocyanin data with literature values is more problematic, since the concentrations of these compounds vary significantly over time, and their values are often reported as relative percentages, as opposed to absolute concentrations. In the few reports that provide quantitative data, large variations are observed even for wines of the same vintage, although the general trend of decreasing concentration with increasing age is observed [46, 56, 57]. The data obtained in this study show the same large variation within vintage, and an inverse relationship between age and concentration of free anthocyanins. However, when comparing wines from the same vintage, certain patterns may be discerned.

In the current data set, the most common vintage was 2003, and the following comments are based on the average values for the anthocyanins determined for all wines of this vintage. The total amount of anthocyanins, as well as the amount of malvidin-3-glucoside (the principal anthocyanin in all these wines) was highest for Ruby Cabernet, followed by Pinotage, while Merlot, Cabernet Sauvignon and Shiraz had similar values. Merlot wines contained the highest amounts of the other anthocyanin-glucosides (delphinidin-, cyanidin-, petunidin- and peonidin-glucoside), while Cabernet Sauvignon contained the highest amounts of malvidin-acetyl-glucoside. The content of acetylated anthocyanins have been shown to distinguish between wine varieties, including Cabernet Sauvignon [26, 27, 32]. The amounts of the derived pigments (vitisin A and the 4-vinyl-phenol adduct of malvidin-glucoside) do not show clear trends in relation to wine age, however, the relative percentage of these compounds (compared to malvidin-glucoside) increased markedly with wine age, in accordance with literature reports [58].

The content of malic- and lactic acids varied more than those of the other acids, no doubt as a result of most, but not all, wines undergoing malolactic fermentation. Malolactic fermentation is a secondary fermentation where lactic acid bacteria convert malic acid to the less acidic lactic acid. The mean content of lactic acid in Merlot wines (1.5 $g \cdot L^{-1}$) was lower than those in the other varieties (≥3.0 g/L). Mean levels for tartaric acid (2.3 $g \cdot L^{-1}$) were higher in Merlot, while acetic acid (0.4 $g \cdot L^{-1}$) was lower in this cultivar, compared to the other four varieties, where the mean values did not differ to any great extent. These differences also can be explained by the lower extent of malolactic fermentation in Merlot wines. Overall, the mean values are in good agreement with numerous reports dealing with wines from other countries [59-64]. Succinic acid levels were consistently in the higher range of reported values. Concentrations of acetic acid were below 1 g/L in all except three wines. One of these wines (a Ruby Cabernet) exhibited a unique acid profile: no tartaric acid was detected, while the same wine contained 6.1 $g \cdot L^{-1}$ lactic acid. This was also reflected in the pH of the wine (4.22), which was the highest for all wines studied (the mean values for red wines pH varied between 3.5 and 3.8).

The levels of the two sugars, glucose and fructose, were below 1 $g \cdot L^{-1}$ in almost all wines, as expected for dry red wines. Within this range, values varied considerably within cultivars, but mean values for each cultivar were roughly similar, with the exception of

Pinotage, which had higher mean values for both compounds. In general, the results are in agreement with literature values [65-69]. Slightly higher trends probably result from the analytical method used as opposed to significant differences in the wines.

11.3.1.2 White Wine

Only the levels of those phenolic compounds quantified in most white wines will be discussed below. For catechin and epicatechin, mean concentrations determined in this study for the cultivars Chardonnay and Sauvignon Blanc correspond to those reported in a previous comprehensive study of wines from all over the world [70]. Mean levels in Chardonnay (7.6 and 5.8 ppm for catechin and epicatechin, respectively) were at the high end of the range reported in this study, and compared with Australian Chardonnays. Also, as reported in the same study, the mean content of these compounds in Sauvignon Blanc (9.4 and 5.9 ppm) are higher than those in the same variety from any other country. Chenin Blanc wines contained lower concentrations of both compounds, particularly epicatechin (5.4 and 1.0 ppm mean values, respectively). The concentrations determined for South African wines also compared favourably with those reported for French white wines [46], but were higher compared to certain Spanish [21] and Canadian [45] wines, and slightly lower compared to different Spanish varieties [71]. p-Coumaric acid content in the analysed wines varied widely, but were in agreement with [71], higher than in Spanish varieties [72] and lower than in Canadian wines [45]. Chenin Blanc had roughly half the mean concentration of this compound (0.8 ppm), compared to the other varieties. The gallic acid content varied significantly within wines of a specific cultivar, but the mean values (2.0-4.7 ppm) correspond to values determined for French wines [46] and are lower compared to Spanish wines [72]. Low levels of ferulic acid (means ~0.4 ppm) and protocatechiuc acid (means 1.2-1.7 ppm) are in rough agreement with literature data [45, 72]. Caffeic acid levels were slightly higher than those of French wines [46], and lower than those of Canadian wine [45]. While the mean concentration of cafta (8.8-14.3 ppm) is comparable to literature data [21, 46], couta levels are lower compared to Spanish wines [21, 72]. Mean levels of trans-resveratrol (0.06-0.10 ppm) correspond to reported values [45, 52, 54, 71]. Cis-resveratrol and the polydatins were quantifiable in only a few white wines, always at

levels below 0.3 ppm, also in agreement with literature. Overall, as expected, white wines contained lower amounts of all phenolic compounds (except ferulic acid, where similar concentrations were found) in comparison to red wines.

The mean values determined for succinic- and acetic acids were similar in all white varieties (0.3-0.4 $g \cdot L^{-1}$), and lower than those reported for red wines. Mean levels of tartaric acid were slightly lower in Chardonnay (1.6 $g \cdot L^{-1}$) compared to Chenin Blanc and Sauvignon Blanc (2.0 $g \cdot L^{-1}$ for both). Amounts of malic- and lactic acids once again reflected the fermentation process. Although only a few white wines had undergone malolactic fermentation, these were mostly Chardonnays. As a result, the mean values for malic and lactic acids are thus lower and higher in Chardonnay (1.8 and 1.0 $g \cdot L^{-1}$, respectively), compared to the other cultivars (~2.5 and 0.3 $g \cdot L^{-1}$, respectively). Roughly the same pattern was observed for citric acid, although the mean levels for all cultivars are higher than found in red wines ($\geq$ 0.2 $g \cdot L^{-1}$). All these values are once again in good agreement with literature reports for white wines [59-63, 64], although tartaric acid levels appear on the low side. pH values were lower in white (mean values between 3.3-3.5) than red wines, also a result of fewer wines undergoing malolactic fermentation.

Mean values for the sugars were higher in white than red wines (1.3-1.6 and 0.7-0.9 $g \cdot L^{-1}$ for fructose and glucose, respectively), but did not show any significant patterns between cultivars. These results correspond to those reported in the literature for white wines [68,72].

11.3.2 Classification of Wines According to Grape Variety

11.3.2.1 Red Wines

11.3.2.1.1 Anthocyanins

ANOVA results for the anthocyanins are presented in table 3 together with mean values obtained for each compound in each of the cultivars. It should be noted that the mean values reported here could be somewhat misleading, since large variations in anthocyanin content for every cultivar were observed. This is a result of a rapid decrease in the

anthocyanin levels with increasing age. Thus varieties for which more young wines were analysed show higher mean amounts. At the chosen significance level of 95%, only the content of cyanidin-acetyl-glucoside and vitisin A did not differ significantly between varieties. Also, there was no significant difference in the anthocyanin composition between Shiraz and Pinotage, or Shiraz and Cabernet Sauvignon.

Variety \ n	Blend 6	Cab Sauv 13	Merlot 10	Pinotage 11	Ruby Cab 10	Shiraz 11	F_{calc}
Cg	0.0	0.1	2.7	0.5	0.5	0.3	5.0
CgAc	0.3	0.6	1.0	1.0	1.0	0.9	1.9
Dg	0.6	2.1	20.2	7.7	14.4	3.8	8.8
DgAc	0.1	0.6	4.8	2.4	3.8	1.1	9.0
DgCm	0.0	0.1	1.6	0.6	2.1	0.5	9.3
Mg	6.8	35.0	125.6	100.0	154.2	58.8	5.9
MgAc	1.0	14.2	37.8	31.2	36.1	18.5	3.1
MgCm	1.1	4.5	18.2	15.1	20.7	9.9	5.7
MgPA	1.0	1.5	2.3	2.4	2.1	2.0	1.8
MgVp	0.2	0.7	0.3	0.4	1.1	0.7	2.6
Png	0.5	1.6	15.1	5.3	5.8	5.2	8.4
PnGAc	0.0	0.8	6.1	3.1	2.4	3.0	6.8
PngCm	0.0	0.2	3.4	1.2	1.4	2.1	6.9
Ptg	0.7	2.7	21.8	12.1	17.9	6.9	8.9
PtgAc	0.2	0.7	5.9	3.5	3.6	1.9	8.8
PtgCm	0.0	0.1	2.0	1.0	2.1	0.9	6.8

Table 11.3: ANOVA results for the anthocyanins in red wines: Mean value for each variety and calculated F values. $F_{crit(5, 58, 0.05)} = 2.4$.

For PCA, four extracted latent variables accounted for 91.4% of the variance. PC1, which accounted for 66.4% of the variance, reflected mostly the properties of malvidin- and petunidin-glucosides and their acetylated and coumaroylated derivatives, but also the acetyl- and coumaroyl derivatives of peonidin- and delphinidin-glucoside. PC2 (10.4%) describes cyanidin-acetyl-glucoside and vitisin A – strangely, those compounds showed not to vary significantly in ANOVA. The remaining glucosides, cyanidin-, peonidin- and delphinidin-glucosides were responsible for the variance explained by PC3 (7.9%), while the vinyl-phenol adduct of malvidin-glucoside created PC4 (6.7%).

In stepwise forward LDA, the following variables were not included in the classification model: delphinidin-, cyanidin-, petunidin- and peonidin-glucosides, cyanidin-(6-acetyl)-3-

From the dendrogram obtained from CA performed on all the anthocyanins three main clusters can be appreciated. The first cluster contained the glucosides: cyanidin-, petunidin-, peonidin-3-glucoside, and delphinidin-3-glucoside together with acylated derivatives of the last three as well as peonidin-(6coumaroyl)-3-glucoside. Members of this cluster form properties of PC1 and PC3 in PCA. The second cluster contains the same variables as PC1: malvidin-3-glucoside, malvidin-(6-acetyl)-3-glucoside and coumaroyl- derivatives of malvidin-3-glucoside, delphinidin-3-glucoside and petunidin-3-glucoside. The third cluster include compounds with low F_{calc} values in ANOVA and associated with PC's explaining a low percentage of the variance: malvidin-3-glucoside-4-vinylphenol, vitisin A and cyanidin-(6-acetyl)-3-glucoside.

11.3.2.1.2 Polyphenols

ANOVA results for the phenolic compounds are summarized in table 11.5. From these results, the content of the following phenolics were shown not to differ significantly (α = 0.05) between cultivars: p-coumaric acid, ferulic acid, gallic acid, myricetin-glucoside, myricetin, protocatechuic acid and couta. Compounds showing large variation between varieties are isorhamnetin, trans- and cis-polydatin, quercetin and cafta. It is interesting that only one of the tartaric acid ester (cafta) shows significant variation between cultivars, as both these compounds have been shown to be influential in differentiating Spanish red wines according to variety [21]. There was no meaningful variance in the phenolic data between the cultivars Cabernet Sauvignon and Ruby Cabernet.

Variety / *n*	Blend 6	Cab Sauv 13	Merlot 10	Pinotage 11	Ruby Cab 10	Shiraz 11	F_{calc}
C	27.0	34.7	57.5	33.8	31.8	42.7	4.8
CA	7.5	8.3	17.4	33.1	7.7	13.8	5.2
CMA	8.1	7.9	5.5	10.6	4.6	10.5	3.1
CPd	0.3	0.2	2.9	0.5	0.7	1.4	12.0
CR	0.7	0.4	1.6	0.5	1.2	0.9	6.3
CTA	21.9	15.8	18.7	45.8	8.1	23.4	9.5
EC	16.4	17.1	37.5	18.6	26.6	27.5	4.9
FA	0.3	0.4	0.5	0.5	0.6	0.4	1.0
GA	27.8	32.4	29.0	28.8	20.6	36.7	2.4
Ir	0.5	1.0	1.5	0.5	0.6	3.5	22.2
IrKg	6.1	9.0	11.7	10.9	10.3	19.8	9.0
Ka	0.2	1.1	2.5	1.0	1.2	1.6	8.0
Mg	2.9	6.7	22.1	18.4	15.4	19.6	3.5
My	3.7	5.1	5.4	4.0	8.0	6.0	1.9
PCA	6.8	5.7	5.8	8.3	7.1	7.5	0.7
PrCA	5.9	4.7	4.6	3.2	3.2	4.3	2.8
Q	4.4	7.4	14.9	4.9	8.3	15.0	13.0
Qg	11.1	14.0	33.5	11.9	18.1	24.6	8.4
Re	1.4	0.7	2.1	0.8	1.2	1.3	4.4
SA	6.3	7.8	4.3	5.2	8.1	5.8	5.4
TPd	1.1	0.3	3.0	0.7	0.5	1.5	13.6
VA	2.4	4.0	4.2	3.8	3.6	5.9	2.9

Table 11.5: ANOVA results for the non-coloured phenolic compounds in red wines: Mean value for each variety and calculated F values. $F_{crit(5, 60, 0.05)} = 2.4$

PCA showed that the first six PC's (selected on eigenvalue 1 criterion) account for 79% of the variation in the polyphenol data. PC1 (27.7% of the total variance) correlates with the flavonoids kaempferol and quercetin as well as their glucosides and another flavonol derivative, myricetin-glucoside. PC2 (14.2%) is described by all four of the stilbenes (cis- and trans-resveratrol, cis- and trans-polydatin), and PC3 (13.0%) by both tartaric esters (figure 11.2). These compounds seem to be influential in explaining the variance in the polyphenol data. The next latent variables and the variables contributing to them are: PC4 (10.8%, isorhamnetin, myricetin, syringic acid and vanillic acid); PC5 (7.5%, the cinnamic acids p-coumaric acid, caffeic acid and ferulic acid); and PC 6 (5.6%, the benzoic acids protocatechuic acid and gallic acid). High correlation was observed in the loadings plot between both isomers of resveratrol, both isomers of polydatin, flavonols and their glucosides, catechins and epicatechin, and to a lesser extent the phenolic acids.

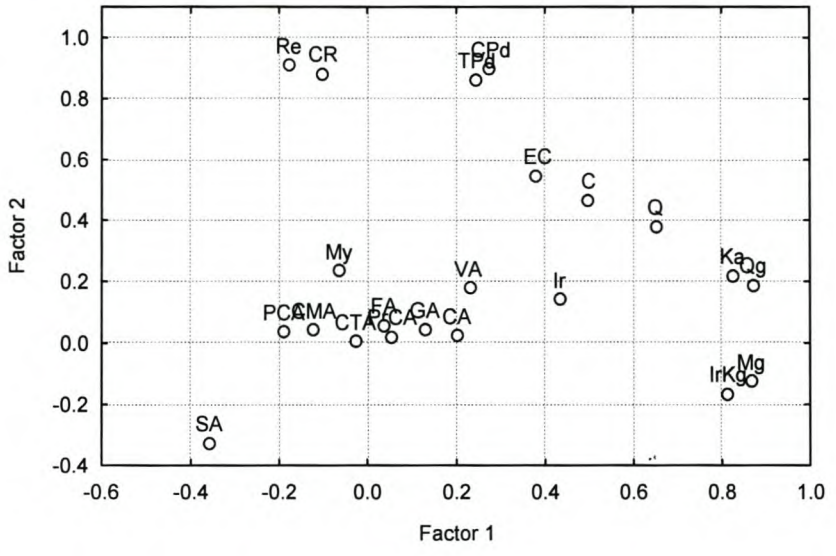

Figure 11.2: Plot of factor loadings on PC1 and PC2 for the phenolic measurements in red wines.

Stepwise forward LDA was performed and a classification function was obtained containing seventeen variables. The classification of the red wines by cultivar is summarized in table 11.6, where it is evident that perfect classification is achieved (this is referred to as recognition, since the training set was classified). The first three roots were found to explain 92.9% of the properties of these wines, and were formed by catechin, epicatechin, caffeic acid, p-coumaric acid, isorhamnetin, isorhametin/kaempferol-glucoside, kaempferol, myricetin, myricetin-glucoside, protocatechuic acid, quercitin, cis-resveratrol, syringic acid, trans-polydatin, cafta, couta and vanillic acid. The following variables were not included in the model: cis-polydatin, ferulic acid, gallic acid, quercetin-glucoside and trans-resveratrol. Some of these compounds were shown not to differ significantly by ANOVA, others were correlated with PC's accounting for limited variation. A scatter plot of the wines in the plane defined by the first two canonical roots is presented in figure 11.3, where it is clear that very good differentiation of the five wine varieties is achieved.

as the flavanols and vanillic acid, compounds responsible for minimal explanation of variance in PCA.

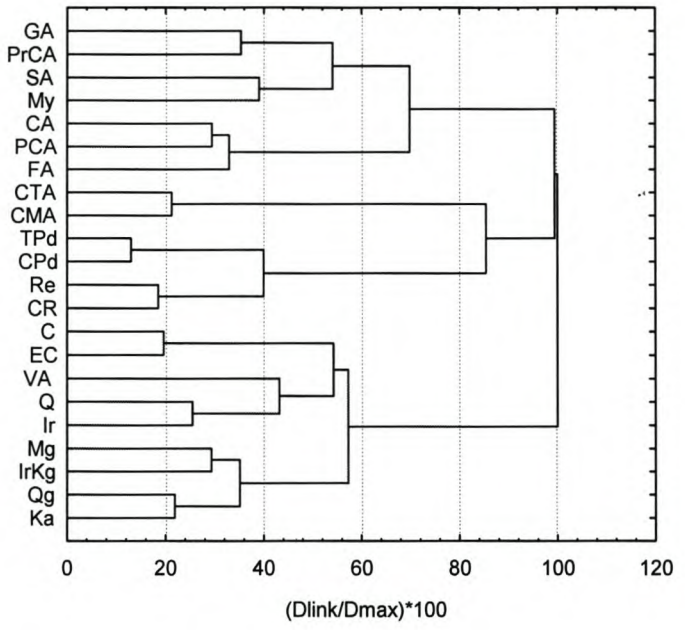

Figure 11.4: Dendrogram obtained for the phenolic data in red wine using Ward's method and Manhattan distances.

11.3.2.1.3 Organic acids, sugars and pH

Of the six organic acids analysed, citric acid showed values below the limit of quantitation in many red wines, and the same was observed for malic acid, in those cases where malolactic fermentation was performed. As a result these compounds were omitted from the statistical investigation of red wines.

ANOVA showed that of the remaining organic acids and sugars, only acetic acid had no significant difference between the means for the different cultivars. There were no significant differences among the values for the following cultivars: Cabernet Sauvignon and Ruby Cabernet, Cabernet Sauvignon and Shiraz, Pinotage and Ruby Cabernet, Pinotage and Shiraz, Ruby Cabernet and Shiraz. In PCA, 74.0% of the total variance was extracted by 3 PC's. PC1 was negatively correlated with acetic and lactic acids, and can thus be

interpreted as describing the fermentation process – although malolactic fermentation leads to the production of primarily lactic acid, acetic acid is an additional product of this process [73]. Both sugars correlate positively with PC2 (sweetness), and tartaric acid and pH are primarily responsible for the variance described by PC3, although their coefficients have opposite signs (tartaric acid is the principal acid responsible for the pH of wines, an increase in its concentration will result in a drop in the pH). This PC is thus related to the fixed acidity of the wines.

Standard LDA using all seven variables provided 72.7% correct prediction for red wine cultivars (table 11.7). In agreement with the ANOVA results, the worst classification was obtained for Shiraz and Pinotage (63.6%).

Group	Percent	Cab Sauv	Merlot	Pinotage	Ruby Cab	Shiraz
Cab Sauv	76.9	10	0	0	2	1
Merlot	80.0	2	8	0	0	0
Pinotage	63.6	0	0	7	3	1
Ruby Cab	80.0	1	0	1	8	0
Shiraz	63.6	2	0	1	1	7
Total	72.7	15	8	9	14	9

Table 11.7: Classification matrix obtained by stepwise forward LDA for the organic acids, sugars and pH for red wines.

CA revealed two main clusters. The first cluster contains the sugars and tartaric acid, compounds that were related in PCA. The second cluster contains the variables pH, acetic and lactic acids, describing the fermentation process. It is interesting that pH is grouped together with lactic and acetic acids, indicating that malolactic fermentation may play a more important role in determining the pH of these wines than the content of tartaric acid (malolactic fermentation results in a decrease in acidity).

11.3.2.2 White Wines

11.3.2.2.1 Polyphenols

In the phenolic data set used for white wines, only eleven variables, detected in most of the wines were used. ANOVA indicated that only epicatechin, cafta and protocatechuic acid are found to differ significantly between wine varieties, and no meaningful variance exists in the phenolic content between Chenin Blanc and Sauvignon Blanc wines.

In PCA, four extracted latent variables cumulatively described 73% of the variance. The compounds catechin, epicatechin, cafta and resveratrol were highly correlated with PC1(29.1%). PC2(22.9%) was correlated with all the cinnamic acid derivatives (p-coumaric acid, caffeic acid, ferulic acid). PC3 (11.1%) was formed by couta and quercetin-glucoside and PC4 (9.8%) by gallic acid. High correlation was observed between p-coumaric acid and caffeic acid, catechin and epicatechin, and to a slightly lesser extent between the two tartaric acid esters.

The results obtained by LDA classification of white wines, as presented in table 11.8, are very good, with only one Chenin Blanc wine misclassified as a Sauvignon Blanc. These results are somewhat surprising considering the ANOVA results. All eleven phenolic compounds were used to calculate two discriminant roots. The score plot for the wines is presented in figure 11.5, and once again the discrimination between cultivars is excellent.

Group	Percent	Chardonnay	Chenin Blanc	Sauv Blanc
Chardonnay	100.0	15	0	0
Chenin Blanc	90.0	0	9	1
Sauv Blanc	100.0	0	0	13
Total	97.4	15	9	14

Table 11.8: Classification matrix obtained by stepwise forward LDA for the polyphenol data for white wines.

and pH contributing maximally. PC3 describes the sweetness and volatile acidity, with sugars and acetic acid displaying high loadings.

Standard LDA was used together with all nine variables to obtain a classification function. Two roots cover 100.0% of all properties of the data. Similar success was achieved as reported for the red wines (73.7%, table 11.9). Classification was better for Chardonnay wines (80.0%) than for Chenin Blanc and Sauvignon Blanc (60.0% and 76.9%, respectively), in agreement with the ANOVA results.

Group	Percent	Chardonnay	Chenin Blanc	Sauv Blanc
Chardonnay	80.0	12	1	2
Chenin Blanc	60.0	1	6	3
Sauv Blanc	76.9	1	2	10
Total	73.7	14	9	15

Table 11.9: Classification matrix obtained by stepwise forward LDA for the organic acids, sugars and pH for white wines wines.

Three clusters can be appreciated from the results of CA. The first cluster groups citric, malic and tartaric acids. The second cluster is formed by the sugars fructose and glucose as well as acetic acid, representatives of sweetness and volatile acidity, respectively. The third cluster contains the variables pH, lactic acid and succinic acid. The groupings for the white wines differ from those obtained for the red wines, but once again roughly resembles the PCA results.

11.3.2.3 Red and White Wines

In the precedent section the training data set (the data set used to derive the classification function in LDA), was used to evaluate the recognition ability of the model. Thus the *posterior* probabilities were calculated. In this section the complete data set for red and white wines is used to classify all the wines according to variety. An example of the classification of an unknown sample is presented, where one sample was left out and LDA

performed on the remaining data set to obtain a classification function, according to which this sample is then classified.

The data matrix used contained the measured amounts for polyphenols, organic acids (except malic and citric acids, for reasons mentioned earlier) and sugars, in all red and white wines except one left out as a test sample (a Chardonnay). The classification function obtained by LDA did not include the variables cis-polydatin, trans-resveratrol, fructose, glucose and pH, and was able to provide correct predictions for 93.6% of the samples (posterior probability). The classification matrix is presented in table 11.10, and the 3D scatter plot in figure 11.6, where the red and white varieties are separated to differently scaled sections for clarity purposes. It is evident that the white wine cultivars are very closely grouped, explaining the worse classification of these wines.

Group	Percent	Cab Sauv	Merlot	Pinotage	Ruby Cab	Shiraz	Chardon.	Chenin Bl.	Sauv Blanc
Cab Sauv	100.0	13	0	0	0	0	0	0	0
Merlot	100.0	0	10	0	0	0	0	0	0
Pinotage	100.0	0	0	11	0	0	0	0	0
Ruby Cab	100.0	0	0	0	10	0	0	0	0
Shiraz	100.0	0	0	0	0	11	0	0	0
Chardonnay	80.0	0	0	0	0	0	12	0	3
Chenin Blanc	90	0	0	0	0	0	0	9	1
Sauv Blanc	84.6	0	0	0	0	0	0	2	11
Total	93.6	13	10	11	10	11	12	11	15

Table 11.10: Classification matrix for the complete data set.

The variance radii for all centroids were calculated using Fisher's F-statistics and the risk α value was determined from F-distribution by probability calculator. From the risk factor the probability, P can be calculated (P = 1 - α). The final result of this procedure is summarized in table 11.12: the "unknown" wine sample is identified as Chardonnay with a 95.2 % probability. When the sample was consequently added to the modelling data set and a new classification was performed, the *posterior* probabilities for this sample were the following: Chardonnay – 97.8%, Chenin Blanc – 0.4 % and Sauvignon Blanc 1.7 %, affirming the improvement of the model by the addition of this sample.

Cultivar	P	α
Cab Sauv	0.000	1.000
Merlot	0.000	1.000
Pinotage	0.000	1.000
Ruby Cab	0.000	1.000
Shiraz	0.000	1.000
Chardonnay	0.952	0.048
Chenin Blanc	0.020	0.980
Sauv Blanc	0.032	0.968

Table 11.12: Calculated prediction probability of unknown cultivar for all varieties

11.4 Discussion

The results of the statistical evaluation of the chemical content of South African wines indicate that, for both red and white wines, the content of the polyphenols demonstrated the best capabilities to classify the wines according to grape variety. This is in general agreement with the work of Soleas et *al.* [45], who reported cultivar-related differences in the phenolic patterns of Canadian wines, although chemometric classification was not attempted. Indeed, De La Presa-Owens et *al.* [21] found this class of compounds most suitable amongst 120 analytical variables for differentiation (by PCA), although not classification, of white wines (obviously, anthocyanins were not included). These authors suggested that the content of phenolic compounds influential in differentiating between

cultivars (cafta and couta), were predominantly influenced by genetics [22]. Phenolic properties (as measured by general parameters such as absorbance readings) also proved useful in the classification of Italian wines by geographical origin [74]. In this study, it is more likely that the differentiation was based on the different cultivars originating from these regions. In another report, some differentiation according to wine variety was attributed to the non-coloured phenolic content, although anthocyanins proved more effective in this regard [30]. Classification of these wines according to variety by DA was very successful, using anthocyanin and polyphenol data. In view of these results, it seems strange that the anthocyanin data did not provide a more effective discrimination of red wines. The broad range of vintages used in the current study may be partially to blame, by obscuring cultivar-related differences. This may be explained in light of the fact that grape anthocyanins decrease rapidly during wine ageing as they are replaced by more stable derived pigments [58], a process that might lead to disruptions of the anthocyanin pattern over extended time period studied here. By normalizing the quantitative results relative to malvidin-glucoside, a more representative classification may be possible, independent of the wine age, although this has not been attempted yet.

In view of these findings, the 100% classification results obtained for wines by non-coloured phenolic content are especially impressive, considering the diverse origins and vintages of the wines. Notwithstanding the well-known fact that the phenolic content of wines undergoes significant changes during wine ageing [75], the phenolic pattern for the analysed wines remained sufficiently characteristic to allow their classification according to variety, in support of reports that suggest the influence of genetics on their composition.

The lack of discriminating power of the acids and sugars is probably explicable in terms of the nature of the analysed wines (all dry wines). The relative content of these compounds in wine reflect more the effects of winemaking practice (specifically fermentation conditions) than those of specific cultivars, which is why they were not influential in this study. A similar conclusion has been reached in a previous study, although in this case shikimic and galacturonic acids (not analysed here) did show some potential for differentiating according to variety [76].

In addition to these findings, it has been demonstrated that a classification function for both red and white wines, based on the content of phenolic compounds and organic acids, is

capable of correct recognition of 93.6% of all wine samples according to variety. This demonstrated the versatility of the classification methods based on chemical content. However, in light of the good classification obtained for red and white wines separately, based only on the phenolic content, practical applications will be based on these last procedures.

Future combination of the results reported here with volatile data obtained for the same wines [77], may further improve their chemical differentiation. Using the combined data, classification of wines according to vintage will also be attempted.

11.5 Conclusions

The successful classification of South African red and white wines using chemical data and multivariate methods has been demonstrated. Analytical data consisting of measured amounts of 11 anthocyanins (red wines only), 16 polyphenols (11 only in white wines), 6 acids, 2 sugars and pH, were obtained for 62 red wines and 38 white wines, of various vintages and origins. The content of these compounds in the wines studied were compared to values reported in the literature.

The phenolic data proved to be best suited for the classification, according to variety, of both red and white wines. Stepwise forward LDA was used to obtain a classification rule in each case, allowing 100.0% and 97.4% correct classification of red and white wines respectively. These results are particularly impressive considering the diverse nature of the wine samples: the classification was possible for wines from various geographical origins and vintages. ANOVA, PCA and CA were used to study underlying properties of the data for each class of compounds. Simultaneous classification of 5 red and 3 white varieties was also demonstrated using phenolic and organic acid data, which allowed correct classification of 93.6% of the wine samples.

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12

Evaluation of LC and CE for the Elucidation of Dyes in Red Wine*

*Published as *"Evaluation of Liquid Chromatography and Capillary Electrophoresis for the Elucidation of the Artificial Colorants Brilliant Blue and Azorubine in Red Wines"*, De Villiers A, Alberts F, Lynen F, Crouch A, Sandra P (2003) Chromatographia 58:393-397

12.1 Introduction

Colouring wine by means of natural grape colours e.g. "grape skin extract" or artificial dyes is prohibited [1-2]. The application of grape skin extracts is rather limited because of the high price of these products. A much cheaper alternative is addition of synthetic azo dyes like brilliant blue and azorubine (figure 12.1) to overproduced white wine that is then mixed with red wine. A number of azo dyes have been shown to represent a potential human health risk, although both the target dyes are allowed in other foodstuffs [2, 3].

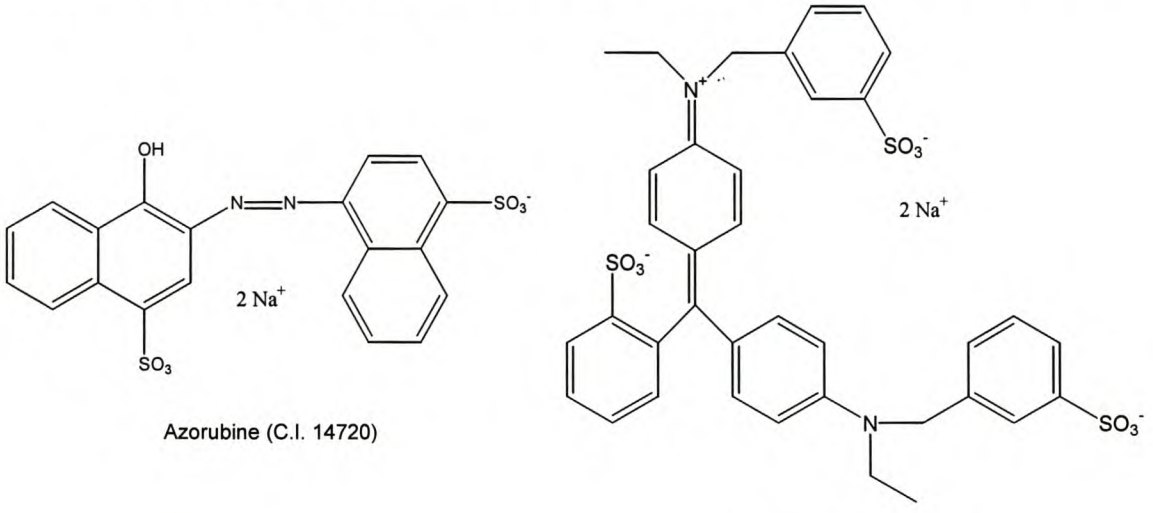

Figure 12.1: Structures of the artificial dyes azorubine and brilliant blue.

In the past the analysis of dyes has been performed using techniques ranging from paper- and thin layer chromatography (TLC) [4-6] to spectrophotometry [7-8], voltammetry [9] and electrophoresis on polyacrylamide [10]. Since the mid-70s liquid chromatography (LC) is the method of choice mainly because of the better sensitivity and resolution LC offers. Reversed phase LC (RP-LC) has been intensively used for these analyses [11-15], but the most popular and powerful method is reversed phase ion pair chromatography (RP-IP-LC) [5, 6, 16-19]. RP-

IP-LC has been applied to a diversity of samples like soft drinks [6], cosmetic products [17], confectionery [19] and forensic fibres [18]. Detection methods employed are UV/VIS and mass spectroscopy (MS). In the case of UV/VIS limits of detection (LOD) of 1-2 $mg \cdot L^{-1}$ (ppm) were reported for a variety of dyes [18], while more recently a detection limit of 12 $\mu g \cdot L^{-1}$ (ppb) for azorubine was obtained [19].

Capillary zone electrophoresis (CZE) [20 – 26], micellar electrokinetic chromatography (MEKC) [27 - 29] and capillary isotachophoresis [30] have also been applied to the analysis of a variety of dyes, including sulfonated azo dyes. The ionic nature of many of these colorants makes these rapid, highly efficient capillary electrophoresis (CE) techniques promising alternatives to LC.

A drawback associated with CE-UV is the low sensitivity resulting from the short path length of the detection cell (the internal diameter of the capillary is normally 50-100 μm). Detection limits for azorubine and brilliant blue are in the range of 1 to 6 ppm, which should be sufficient for the analysis of most wines contaminated with synthetic dyes. Better sensitivities have been described by applying large bore capillary tubes [22], stacking techniques [20] and coupling to electrospray ionisation MS (CE-ESI-MS) [21, 25, 26].

In most studies the composition of the samples was relatively simple. Either standard solutions or extracts of products containing few interfering components were analysed. The analysis of synthetic dyes in wine, however, is a more challenging task. In order to allow spectral identification, good separation of the dyes from many interfering compounds, either prior to or during the analysis, is a prerequisite. The polyphenols present in wine are responsible for most of the problems associated with the determination of dyes in wine. This is mainly because of the diversity of structures and characteristics of these compounds, varying from the phenolic acids to the complex polymeric structures known as tannins [31]. The phenolic acids possess similar characteristics to the sulfonated dyes and absorb strongly in the UV-region of 200-340 nm. As a consequence, these acids have to be separated or fractionated from the dyes to allow spectral confirmation of the dyes. Another group of polyphenols, the anthocyanins, absorb in the region 500-600 nm at wine pH. This means that the use of these wavelengths for selective

detection and quantitation of the dyes can only be warranted if sufficient chromatographic resolution between these polyphenols and the dyes is obtained.

It can be supposed that wines contaminated with artificial colorants will contain these compounds at concentrations in the ppm range, since it is only in that range that the dyes will have any effect on the colour of wine. However, in this study some of the samples analysed were severely diluted with uncontaminated red wine. This means that dyes had to be detected at much lower concentrations (less than 0.1 ppm) than could possibly have any effect on the colour of the wine.

To the best of our knowledge this problem has not been dealt with before. An LC and a CE method, combined with suitable sample preparation strategies, are described allowing the sensitive and reliable analysis of the synthetic dyes azorubine and brilliant blue in red wine samples.

12.2 Experimental

12.2.1 Materials

Acetonitrile HPLC grade was from ROMIL (Cambridge, UK). All solvents were filtered through 0.45 μm HV filters before use (Millipore Corporation, Bedford, MA). Azorubine (Acid Red 14, C.I. 14720), brilliant blue FCF (Erioglaucine, C.I. 42090), tetrabutylammonium bromide (TBAB), decyltrimethylammonium bromide (DTAB), cetyltrimethylammonium bromide (CTAB), tri-*n*-octylamine, potassium dihydrogenphosphate and phosphoric acid were purchased from Sigma-Aldrich (Atlasville, South Africa). Buffers for CE were filtered through disposable syringe filters (Millex HN 0.45μm Nylon, Millipore). The SPE cartridges (3 mL, 500 mg phase) tC18 Sep-Pak were purchased from Waters Corporation (Milford, MA, USA).

12.2.2 Instrumentation

LC analyses were carried out on an Alliance 2690 equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). Data analysis was done using Millenium[32] Chromatography Manager software (Waters). RP-LC experiments were performed on a Waters Nova-Pak C18 column (15 cm × 3.9 mm i.d. × 5 μm particles), while the ion pairing separations were performed on a Macherey-Nagel (Düren, Germany) C18 column (12.5 cm × 4 mm i.d. × 5 μm particles). All experiments were performed at a flow rate of 1 $mL \cdot min^{-1}$. The injection volume was 30 μL and the column temperature was kept constant at 27 °C. Detection was at 515 and 625 nm for azorubine and brilliant blue, respectively. The polyphenols were monitored at 280 nm. For the RP-LC and the RP-IP-LC experiments binary gradients were used. The compositions are given in table 12.1. The pH of the aqueous portion of the eluent was kept at 4.7. The gradient details are specified in the appropriate sections in the text.

Ion-pairing reagent	Amount	$CH_3CN:H_2O$ (A)	$CH_3CN:H_2O$ (B)	KH_2PO_4 (in H_2O)[a]
-	-	50:50	5:95	2.5 g/L
TBAB	8 mM	50:50	5:95	2.5 g/L
DTAB	8 mM	60:40	5:95	2.5 g/L
CTAB	0.3 mM	60:40	10:90	2 g/L

[a]The aqueous phase was adjuste to pH 4.7

Table 12.1: Compositions of the mobile phases used in RP-IP-LC experiments.

The CE analyses were performed on a HP^{3D} CE capillary electrophoresis system equipped with diode array detection from Agilent Technologies (Waldbronn, Germany). Bare fused silica capillaries (Composite Metal Services Ltd., Worcester, UK) with an internal diameter of 75 μm and a total length of 48.5 cm were used. In the final conditions a 50 mM phosphate buffer was prepared by dissolving the appropriate amount of KH_2PO_4 in water and adjusting to pH 4 with 0.1 M H_3PO_4. Injection was done by applying 50 mbar pressure for 8 s (~ 64 nL injection

volume). Detection was at 515 and 590 nm for azorubine and brilliant blue, respectively, while other sample components were monitored at 214 nm.

12.2.3 Sample Preparation

12.2.3.1 Liquid-Liquid Extraction for HPLC Analysis

100 mL wine was extracted by shaking for 20 min with 20 mL chloroform containing 0.46% (v/v) tri-*n*-octylamine. After centrifugation for 5 min at 5000 rpm, the organic layer was removed and evaporated to dryness on a rotavapor. The extract was then washed by adding 2 mL hexane and agitating in a vortex mixer, followed by centrifugation and the removal of the hexane layer. The residue was re-dissolved in 1 mL of chloroform and back transferred to the aqueous phase by adding 1.5 mL of a 1 M $NaClO_4$ solution, vortexing and finally centrifuging the mixture. The top (aqueous) layer was recovered for analysis.

12.2.3.2 Reversed Phase SPE for CE Analysis

tC18 SPE cartridges were conditioned with 2.5 mL methanol followed by 2.5 mL water (pH 7). 2.0 mL wine samples were loaded onto the cartridges. The acidic polyphenols were not retained at this pH and were removed with 2.5 mL water (pH 7). The retained dyes together with the more hydrophobic polyphenols were eluted with 2 mL methanol.

12.3 Results and Discussion

12.3.1 LC Analysis of Dyes in Wine

Initially, samples prepared by liquid-liquid extraction were analysed by RP-LC. Figure 12.2 shows a chromatogram of a blank red wine spiked with 30 ppb of both dyes. It can be seen that under these conditions the dyes are co-eluting with the bulk of the polyphenols co-extracted

with the dyes (visible at 280 nm). The anthocyanins absorb strongly in the region of 515 nm at the pH of the mobile phase. The broad hump between 8 and 14 minutes is caused by the elution of the polymeric tannins and anthocyanins, which cannot be resolved. Moreover, certain non-coloured polyphenols co-elute with the dyes and since these compounds absorb strongly at 200-300 nm, the spectra obtained for the dyes could not be used for identification purposes. The spectra recorded for brilliant blue and azorubine under these conditions and comparison with library spectra are given in figure 12.2.

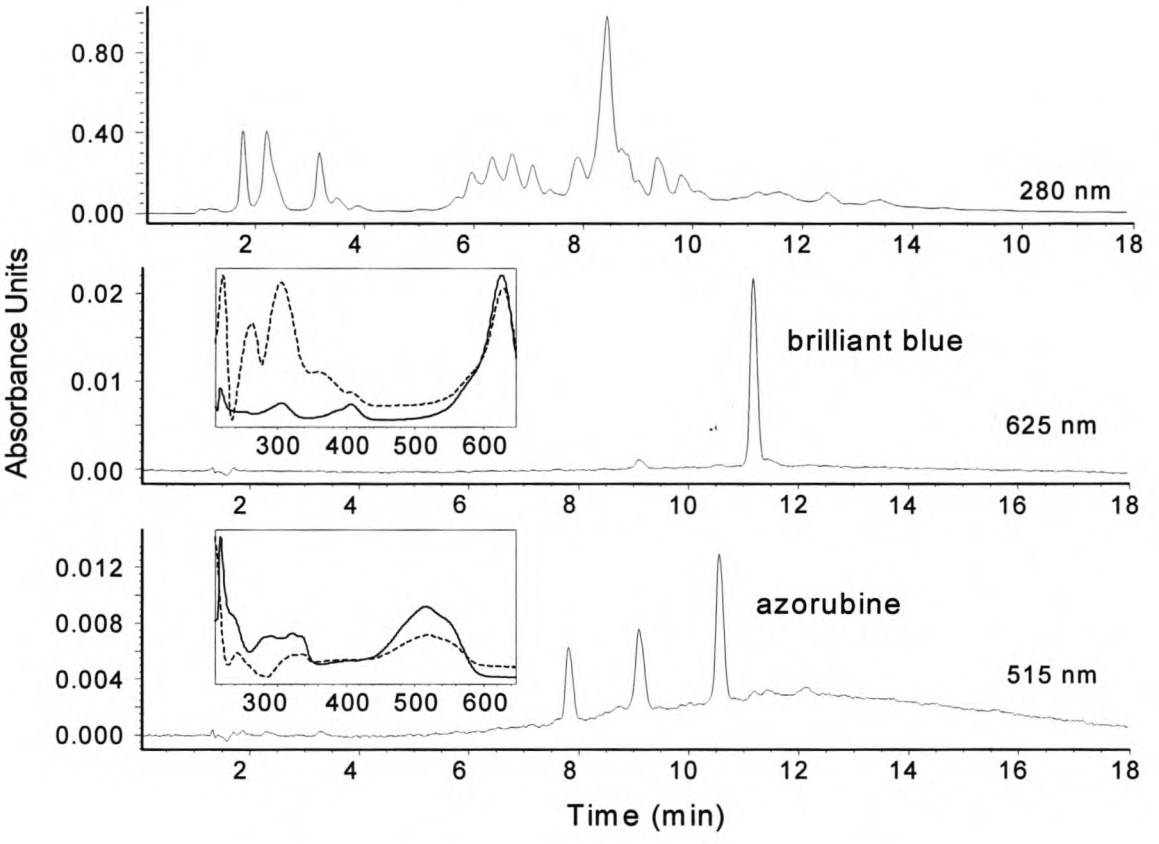

Figure 12.2: RP-LC analysis of the dyes in wine at 30 ppb. Inserts: comparison between UV spectra of dye standards (solid line) and dyes in sample (dotted line).

In an attempt to separate the dyes from the interfering polyphenols the use of RP-IP-LC was investigated. Ionic surfactants possessing alkyl substituents were added to the mobile phase at

concentrations below their critical micellar concentration (CMC). Through ionic interaction of the cationic surfactants with the acidic dyes a greater apolar character is imparted to these solutes, thereby increasing their retention on a reversed phase column. Since most of the polyphenols are neutral at the pH of the buffer (4.7), only the acidic dyes should interact with the surfactants and as a result their retention should be selectively increased. Initially tetrabutylammonium bromide (TBAB) was added in varying amounts to the mobile phase eluents (8 mM seemed optimal). Under these conditions the dyes could be separated from the anthocyanins but not from the rest of the polyphenols. The latter caused spectral interferences hindering the identification of the dyes when present in low concentrations. When TBAB was replaced by decyltrimethylammonium bromide (DTAB), the dyes could well be separated from the polyphenols. This is illustrated in figure 12.3. This method ensured that the bulk of the polyphenols eluted before the dyes. The same sample was analysed in figures 12.2 and 12.3. The UV spectra could now be used to identify the dyes even at these low concentrations of 30 ppb. Similar results were obtained with cetyltrimethylammonium bromide (CTAB), but lower surfactant concentrations were required due to its higher hydrophobicity (table 12.1).

Liquid-liquid extraction followed by RP-IP-LC proved to be a successful approach for samples containing 10 and 20 ppb for azorubine and brilliant blue, respectively. Good spectral identification and quantification could be performed under these conditions. This method proved sufficiently sensitive for the analysis of all wine samples.

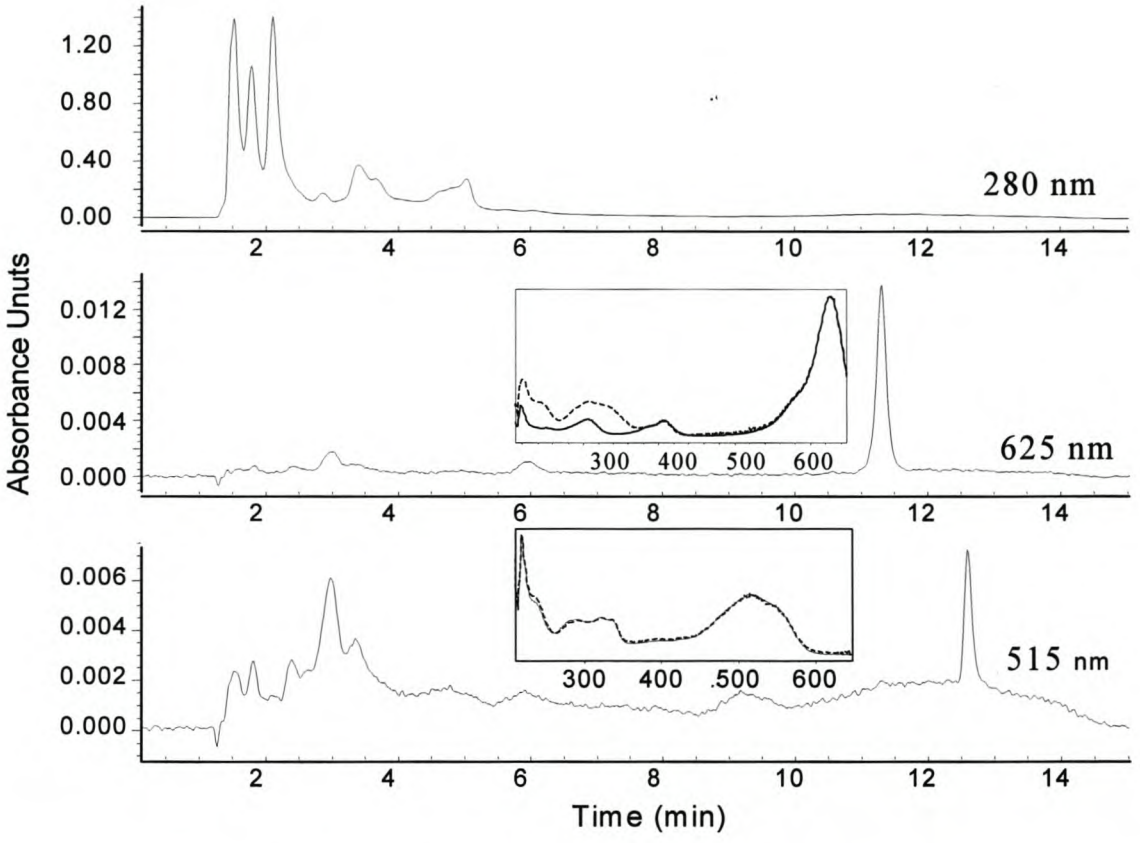

Figure 12.3: RP-IP-LC analysis of the dyes in wine at 30 ppb. Inserts: comparison between UV spectra of dye standards (solid line) and dyes in sample (dotted line).

In order to investigate if spectral identification could be performed at even lower dye concentrations, the pre-concentration and isolation of the dyes by solid phase extraction was attempted. This included reversed phase ion pair- and anion exchange SPE. Both sample preparation techniques offer the advantages of speed and simplicity coupled to high concentration factors and recoveries. However, this approach appeared unsuccessful because the wine components co-eluting with the dyes were also pre-concentrated with both SPE procedures.

Because artificially coloured wines can generally be expected to contain dyes in the ppm level it was also investigated if samples could be analysed by direct injection of filtered wine using

the ion pairing LC method. An example is shown in figure 12.4. A 10 ppm spiked wine sample was analysed using the CTAB ion-pairing method.

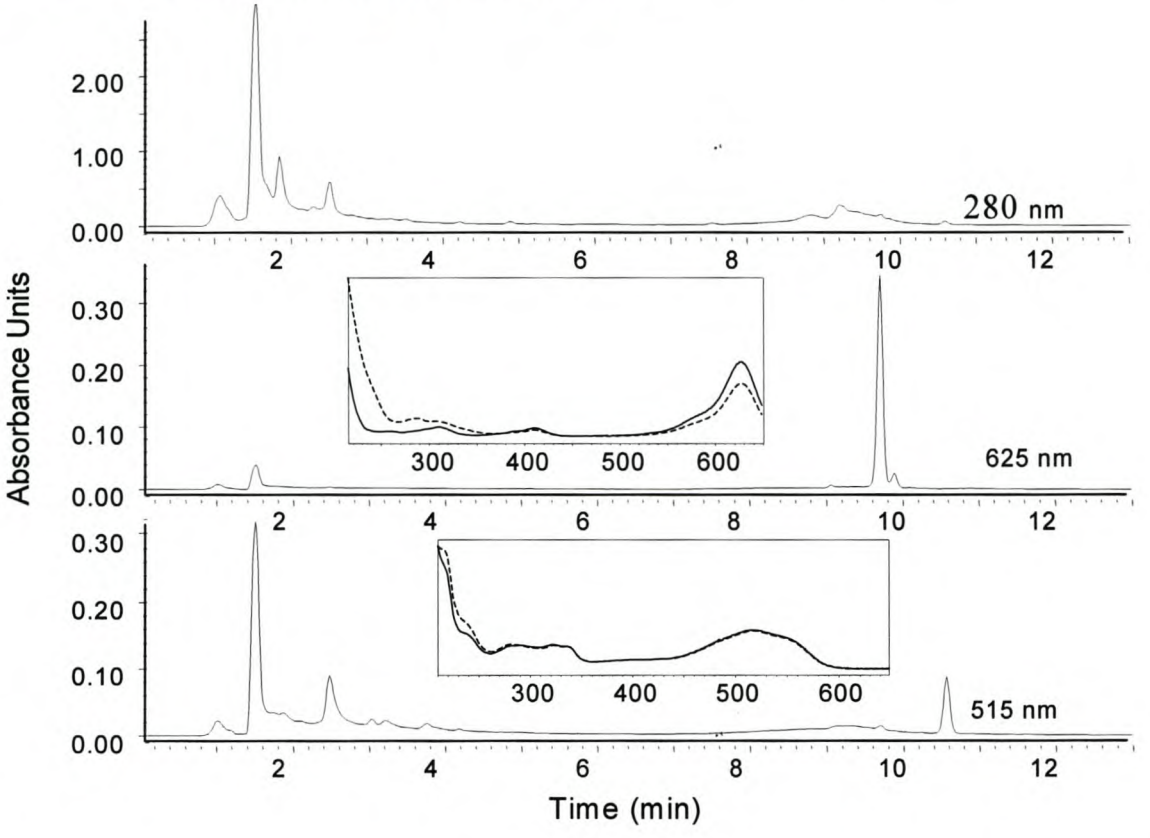

Figure 12.4: Direct RP-IP-LC analysis of the dyes in wine at 10 ppm. Inserts: comparison between UV spectra of dye standards (solid line) and dyes in sample (dotted line).

12.3.2 CE Analysis of Dyes in Wine

The applicability of alternative separation mechanisms offered by capillary zone electrophoresis (CZE) for the analysis of dyes in wine was investigated and compared to the LC analyses discussed in the previous section. By working at sufficiently low enough pH (4.0) it is ensured that the pigments in wine, the anthocyanins, are in the flavylium cationic form (the only form that absorbs in the 500-600 nm region). Under these conditions it should be possible to separate them from the negatively charged dyes. The separation was achieved by reversing

the polarity of the applied voltage. Since the electro-osmotic flow (EOF) in a bare fused silica capillary is much reduced at pH 4.0, the electrophoretic mobilities of the negatively charged dyes are greater than the EOF and the dyes elute at the anode. The anthocyanins migrate in the same direction as the EOF – towards the cathode – and thus these compounds do not interfere with the dyes.

It was found that, using these conditions, the phenolic acids were the only polyphenols detected upon direct injection of wine. This meant that 515 nm and 590 nm could be used as wavelengths for selective detection of the dyes. However, unambiguous identification of the dyes by their UV spectra is not guaranteed. For this reason alternative methods of sample preparation, applicable to CE, were investigated. The SPE procedure described in the experimental part was therefore developed. This procedure allowed complete removal of the acidic polyphenols from the samples. The result is shown in figure 12.5, where total elimination of interference by the phenolic acids is evident. The obtained spectra can therefore be used for identification purposes. The recovery of the sample preparation procedure was found to be excellent i.e. larger than 98% (RDS% 3.6 for $n = 3$).

An attempt was made to increase the sensitivity by on-capillary sample concentration using methods such as field amplified sample injection (FASI) [28, 32] and large volume sample stacking (LVSS) [20]. Although both techniques provided effective pre-concentration of the dye standards, neither could be successfully employed to analyse real wine samples, where matrix effects and interferences rendered results unusable. A more successful sample concentrating approach was obtained by simply evaporating 5 mL of the SPE eluent to dryness and re-dissolving it in 200 µL methanol. Under these conditions the dyes could be spectrally identified at concentrations down to the 0.5 ppm level.

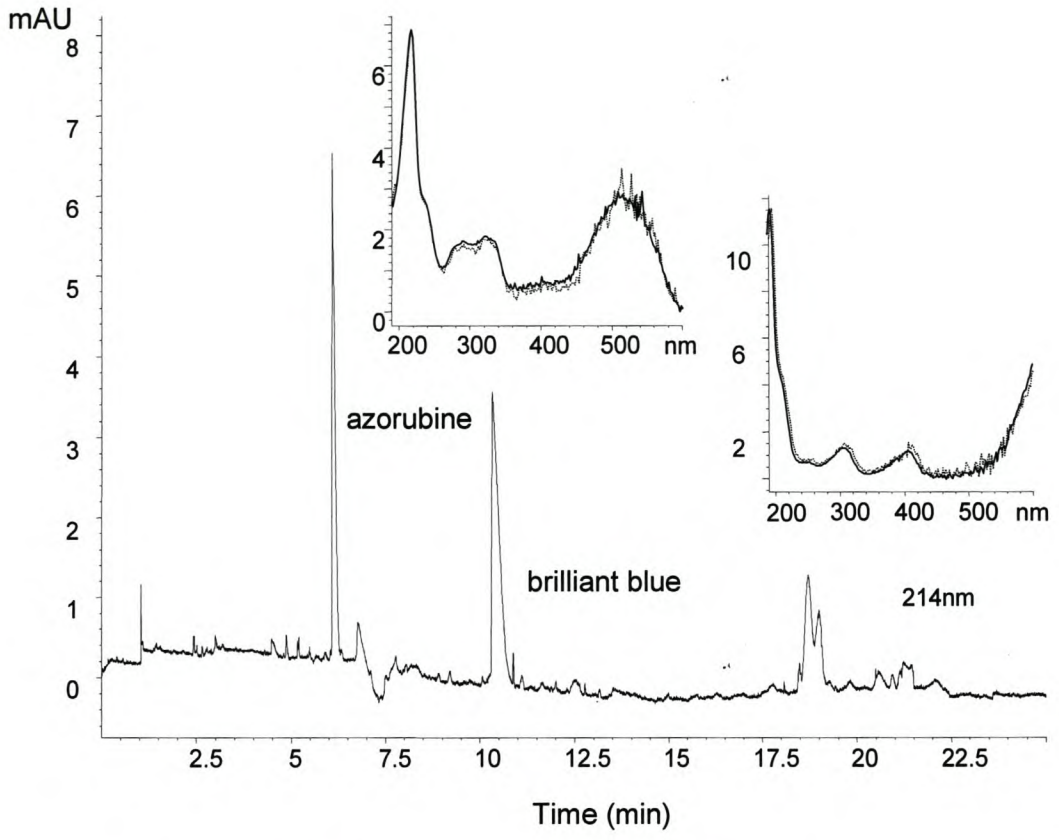

Figure 12.5: CE analysis of the dyes in wine at 10ppm. Inserts: comparison between UV spectra of dye standards (solid line) and dyes in sample (dotted line).

12.3.3 Quantitation of the Dyes

In the case of direct analysis of dyes at the ppm level, external calibration using the dye standards was used for both LC and CE. For the LC analysis no sample preparation is required at this level. The calibration graphs were linear in both cases in the range 1-100 ppm (5 levels), with correlation coefficients higher than 0.999.

For diluted samples analysed by LC after liquid-liquid extraction, standard addition at three levels (25, 50 and 100 ppb) was performed ($R^2 > 0.982$). From the graphs the amounts

originally present in a suspicious red wine were determined as 74 ppb for azorubine and 22 ppb for brilliant blue.

For the purpose of this study, namely spectral confirmation required, the limit of detection (LOD) for direct LC analysis were ~ 1 and 5 ppm for azorubine and brilliant blue, respectively. For CE after SPE clean-up the values are ~ 5 ppm for both dyes. For diluted wine samples, where liquid-liquid extraction was performed, the LOD were ~ 10 and 20 ppb for azorubine and brilliant blue, respectively.

12.4 Conclusion

Two complementary separation techniques suitable for the analysis of the artificial colorants azorubine and brilliant blue in wine have been evaluated. A reversed-phase ion-pairing HPLC method was developed where interference by polyphenols could be eliminated in order to identify the colorants by their UV-spectra. In combination with liquid-liquid extraction this method proved useful to identify the dyes down to the 10-20 ppb range. In the CE separation method the interfering phenolic acids had to be removed by solid phase extraction to allow spectral identification. Both methods proved reproducible and easy to implement.

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13

Stir Bar Sorptive Extraction-Liquid Desorption for the Analysis of Hop Bitter Acids in Beer by MEKC*

*Published as *"Stir Bar Sorptive Extraction-Liquid Desorption Applied to the Analysis of Hop Derived Bitter Acids in Beer by Micellar Electrokinetic Chromatograph"*, De Villiers A, Vanhoenacker G, Lynen F, Sandra P (2004) Electrophoresis 25:664-669

13.1 Introduction

The bitter taste of beer is derived from hop (*Humulus lupus* L.) or hop extracts added to the wort during brewing [1]. In the boiling process the hop α-acids or humulones are converted into the iso-α-acids or isohumulones. These products are not only responsible for the bitter taste of beer, but also exhibit bacteriostatic properties and furthermore play an essential role in the foam stability of beer as well as in the formation of off-flavours like the sunstruck flavour [2-4]. Reduced iso-α-acids such as rho-iso-α-acids and tetrahydro-iso-α-acids are often added in the brewing process to enhance the light stability of the taste and foam stability. LC is intensively used for the analysis of α- and β-acids in hops [5-7] and iso-α-acids in beer [2,8]. Problems related with interaction of the solutes with trace metals in the chromatographic system resulting in poor resolution and quantitation have been reported [2,8]. Electrodriven separation techniques such as micellar electrokinetic chromatography (MEKC) [9,10], microemulsion electrokinetic chromatography (MEEKC) [11] and capillary electrochromatography (CEC) [12] have been evaluated for the analysis of the hop acids while the iso-α-acids were successfully separated by MEKC [13, 14]. For the analysis of the iso-α-acids in beer, a pre-concentration step is normally performed, although direct injection of beers with stacking has been described [15]. Enrichment is commonly carried out by liquid-liquid extraction or by solid phase extraction on reversed phase material [13, 15, 16]. Recently a new solventless extraction method named stir bar sorptive extraction (SBSE) has been described [17]. SBSE is normally combined with thermal desorption (TD)-capillary GC (cGC) analysis and the sensitivity and simplicity of SBSE has been demonstrated for the analysis of volatiles and semi-volatiles in a variety of sample matrices like water [18], beverages [19], sperm [20], etc. SBSE, however, can also be combined with liquid desorption (LD) and this opens perspectives for the analysis of thermally labile compounds. Popp et *al.* used SBSE-LD for the determination by LC-fluorescence detection of polyaromatic hydrocarbons (PAH) in water samples [21] and Sandra et *al.* used SBSE-LD to confirm SBSE-TD-cGC results for the determination of fungicides in wine by LC-

MS [22] and Harms et *al.*[23] presented the first application of SBSE-LD for the profiling of iso-a-acids in beer. The features of SBSE have recently been reviewed [24].

In this contribution, the use of SBSE-LD for quantitative analysis of iso-α-acids and for the elucidation of reduced iso-α-acids in various beers by MEKC is described. The structures of the investigated compounds are presented in figure 13.1. The SBSE sorption and desorption processes are discussed. The iso-α-acid content of several beers was determined and compared with results obtained with HPLC using direct beer injection.

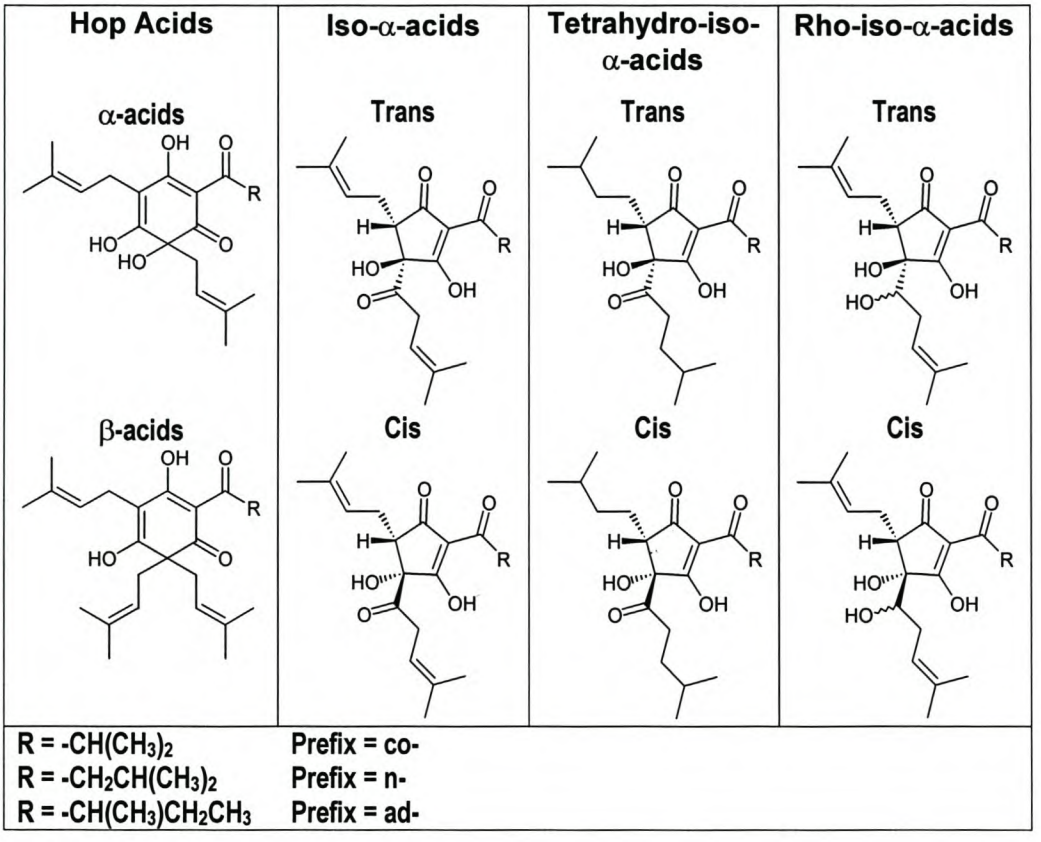

Figure 13.1: Structures of the investigated hop compounds.

13.2 Materials and Methods

13.2.1 Chemicals and Reagents

Na_2HPO_4, HCl and NaOH were from Merck (Darmstadt, Germany), sodium dodecylsulphate (SDS) from BDH Chemicals Ltd. (Poole, UK) and $MgSO_4$ from Saarchem (Cape Town, South Africa). HPLC grade acetonitrile was purchased from Romil (Cambridge, UK). The HPLC mobile phase was filtered through a 0.45 µm HV filter (Millipore Corporation, Bedford, MA, USA) and the buffer for the MEKC analyses was filtered through a disposable syringe filter (Millex HN 0.45µm Nylon, Millipore Corporation, Bedford, MA, USA). Polydimethylsiloxane (24 µl PDMS) coated stir bars (commercialized as Twister^{TM}) were supplied by Gerstel (Mülheim a/d Ruhr, Germany). Beers were bought at local stores, kept at room temperature and freshly opened prior to analysis. The pre-isomerised hop extract containing iso-α-acids was obtained from Hopstabil GmbH (Wolnzach, Germany). The rho-iso-α-acids (DCHA-Rho, ICS-R1) were purchased from Labor Veritas (Zürich, Switserland) and the tetrahydro-iso-α-acids from Kalsec (Kalamazoo, MI, US).

13.2.2 Instrumentation

HPLC analyses for the investigation of the sorption/desorption processes were carried out on an Alliance 2690 Separations Module equipped with a 996 photodiode array detector (Waters Corp., Milford, MA, USA). The data analysis was done using Millenium^{32} Chromatography Manager software (Waters). The column was a Nucelosil C18 "Hop" column from Macherey-Nagel (Düren, Germany), 25 cm in length with an internal diameter of 4 mm and packed with 5 µm particles. The mobile phase consisted of acetonitrile/water/H_3PO_4/ $MgSO_4$ in ratio 500/500/20/6 (w/w) at a flow rate of 1.5 mL·min^{-1}. Detection was performed at 270 nm and the injection volume was 30 µL, except for the

sorption kinetics samples, where 10 µL was injected. The same beer samples were analysed by direct beer injection-HPLC. Full details of the method are described elsewhere (Vanhoenacker et *al.*, personal communication, paper submitted to J. Chromatogr. A).
MEKC analyses were performed on a $HP^{3D}CE$ Capillary Electrophoresis System equipped with DAD (Agilent Technologies, Waldbronn, Germany). An extended light path bare fuse silica capillary (Agilent Technologies, Waldbronn, Germany) with an internal diameter of 50 µm and a total length of 64.5 cm (effective length 56 cm) was used. The buffer consisted of 40 mM phosphate (pH 10.2 with NaOH) containing 80 mM SDS. Injection was performed hydrodynamically at 50 mbar for 10 s for the SBSE enriched samples and for 15 s for direct injection of beer. The separation voltage was 25 kV and detection was performed at 255 nm. The capillary temperature was set at 25°C. Between runs the capillaries were conditioned by flushing with NaOH (0.1 N) for 2 min, water for 1 min and buffer for 3 min, consecutively.

13.2.3 Sample Preparation

Different sample preparation parameters i.e. for sorption and desorption, were studied as outlined in the Results and Discussion section. The final SBSE-LD-MEKC procedure is as follows: 20 mL beer is poured in a headspace vial of 40 mL and 1 mL 1 M HCl is added. A stir bar containing 24 µL PDMS is introduced and the sample is stirred at 1500 rpm and at 25°C for 60 min. The stir bar is dipped in bidistilled water and dried on a tissue paper. The stir bar is then placed in 2 mL acetonitrile and stirred at 1500 rpm and 25°C for 60 min. A 1 mL portion is taken and evaporated to dryness under a nitrogen stream. The residue is dissolved in 0.5 mL bidistilled water and the sample is injected.

13.3 Results and Discussion

13.3.1 Investigation of the Sorption Process

In the first instance the sorption and desorption kinetics were studied. HPLC was hereby used because of its greater sensitivity compared to MEKC, although only five peaks for the six iso-α-acids are recorded. Cis-humulone and trans-adhumulone are co-eluting under the HPLC conditions applied.

The sorption step was investigated as follows. The stir bar was added to 20 mL beer acidified with 1 mL 1 M HCl. Magnetic stirring at different time intervals took place in a closed vial covered with aluminum foil. After each sorption step, the stir bar was removed and rinsed with water before being placed in 2 mL acetonitrile for 60 minutes (stirred as for sorption). Thirty microliter of this desorption liquid was then directly injected into the HPLC. Only one stir bar was used which was regenerated between procedures by placing it in a Soxhlet extractor containing ~200 mL acetonitrile for a minimum of 2 hours. The results are summarised in figure 13.2.

Equilibrium between the PDMS coating and the aqueous phase has not been reached after 120 minutes. The long equilibration time can be accounted for by the slow diffusion rates of these relatively large molecules into the PDMS. Since the diffusion time is proportional to the square of the film thickness of the coating, the equilibration time can be greatly reduced by decreasing the thickness of the coating. The short equilibration times encountered when using Solid Phase Micro Extraction (SPME) demonstrates this approach [25]. However, the loss of sensitivity that would result from a thinner coating would make the analysis of the iso-α-acids impossible. For reasons of convenience 60 minutes was chosen as the optimum sorption time, since acceptable results were obtained in terms of reproducibility (see further).

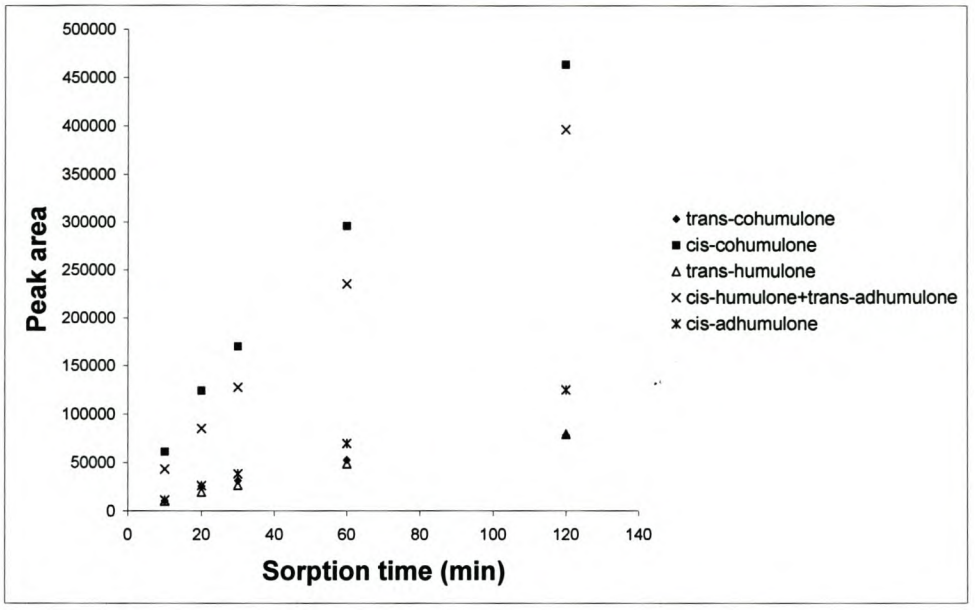

Figure 13.2: Sorption graph for beer iso-α-acids.

Comparison was also made between using "dry" twisters and twisters soaked in iso-octane and acetonitrile before sampling. One stir bar was used in these studies and it was regenerated between experiments by thermal desorption (2 h 300°C). By swelling of the PDMS in iso-octane, diffusion should be faster while by doping with acetonitrile the polarity should be higher. Although higher recoveries were obtained compared to dry sampleing for the same period of time (*ca.* 1.1 for acetonitrile and 1.8 for iso-octane), reproducibility was lower.

13.3.2 Experimental Determination of log P Values for the Iso-α-acids

At equilibrium conditions, the quantity extracted in the stir bar (m_{PDMS}) is given by:

$$m_{PDMS} = m_0 \frac{K_{PDMS/beer} / \beta}{1 + K_{PDMS/beer} / \beta} \qquad (1)$$

in which m_0 is the quantity of bitter acids present in beer, K is the distribution or partitioning coefficient of the iso-α-acids between PDMS and beer, and β is the phase ratio PDMS/beer. In order to have an idea on the recovery, $K_{PDMS/beer}$ can be replaced by the octanol-water coefficient $K_{o/w}$ [26], and since the volumes of the PDMS coating (24 µL) and the aqueous phase (25 mL) are known, β can be calculated. To determine the log $K_{o/w}$ (log P) values experimentally, SBSE together with HPLC analysis was employed. To simulate PDMS/water conditions, an isomerised hop extract was dissolved in methanol and diluted to a level of 0.5% methanol. The sample was acidified as for beer before adding an unused stir bar. The sample was stirred for 8 h in a completely filled vial (25 mL) covered with aluminum foil. 8 h was estimated as sufficient sorption time for equilibrium to be established from a curve fit to the sorption data summarised in figure 13.1. 30 µL was injected prior to and after stirring. From these injections the ratio m_{PDMS}/m_0 and thus the log P (=log $K_{o/w}$) values for the hop acids could be calculated. The results are summarised in table 13.1. Loss of sample components observed in a control sample was taken into account in the stir bar calculation. The values obtained using this method were compared to the values calculated using the software program KowWIN [27]. It should be noted that since the log P values for these compounds could not be obtained from either the ACD/Labs Log P or KowWIN databases, they had to be calculated using the KowWIN program. These estimations are based on the relative numbers of carbon, hydrogen and oxygen comprising the molecule of interest. As a result identical values are obtained for the isomers, as well as for humulone and adhumulone, since these compounds have identical molecular formulae. As is evident from table 13.1, the experimentally determined values are consistently smaller, though of comparable magnitude. Here the values of different isomers seem to be similar, although increasing slightly in the order humulone < cohumulone < adhumulone. It is interesting to note that the experimentally calculated log P value of trans-humulone is smaller than those of either cohumulone isomer, although the latter molecules possess one less carbon atom.

Using the experimentally calculated log P values and the known phase ratio β, the theoretical recovery of hop acids from beer (with the assumption that $K_{PDMS/beer} \approx K_{o/w}$) was determined as 45 % (for log P = 2.9). It should be noted that these recoveries are not obtained under the conditions employed in this study for the analysis of beer (i.e. 60

minutes sorption time), since equilibrium has not been established. Under equilibrium conditions, however, these recovery values can be used to determine the amount of hop acids originally present in the beer (Q_o). In this study sufficient recoveries and reproducibility were obtained for a sorption time of 60 minutes, while the iso-α-acids could be quantified by determination of the concentration factor for an isomerised hop extract of known concentration (see section 13.3.4).

Acid	log P Calculated (KowWIN)	log P Experimental (stir bar)
trans-cohumulone	3.0	2.9
cis-cohumulone	3.0	2.9
trans-humulone	3.5	2.8
cis-humulone+trans-adhumulone	3.5	2.9
cis-adhumulone	3.5	3.0

Table 13.1: Comparison between calculated and experimentally determined log P values for the iso-α-acids.

13.3.3 Investigation of the Liquid Desorption Process

In order to study the desorption step, the sorption time was kept constant at 60 minutes. Acetonitrile was chosen as desorption solvent because of its efficiency in desorbing relatively polar compounds from the stir bar without causing excessive swelling of the PDMS coating. Sorption took place in acidified beer as described previously. After removal and rinsing, the stir bar was placed in 2 mL acetonitrile. After incremental time fractions 10 μL of the desorption liquid was removed and injected directly into the HPLC. From the desorption graph in figure 13.3 it can be seen that virtual equilibrium between the 2 phases has been reached after 60 minutes, and this was chosen as the optimum desorption time in subsequent analyses.

In further experiments (data not shown) it was demonstrated that the desorption equilibration process could be speeded up, by either heating or sonication of the desorption liquid. This is a result of increased diffusion rates at elevated temperatures, as well as possible temperature dependence of the relevant distribution coefficients. In the case of SBSE-LD-MEKC analysis of beer, sensitivity and reproducibility were found to be

adequate, and sorption and desorption were subsequently performed at room temperature without sonication.

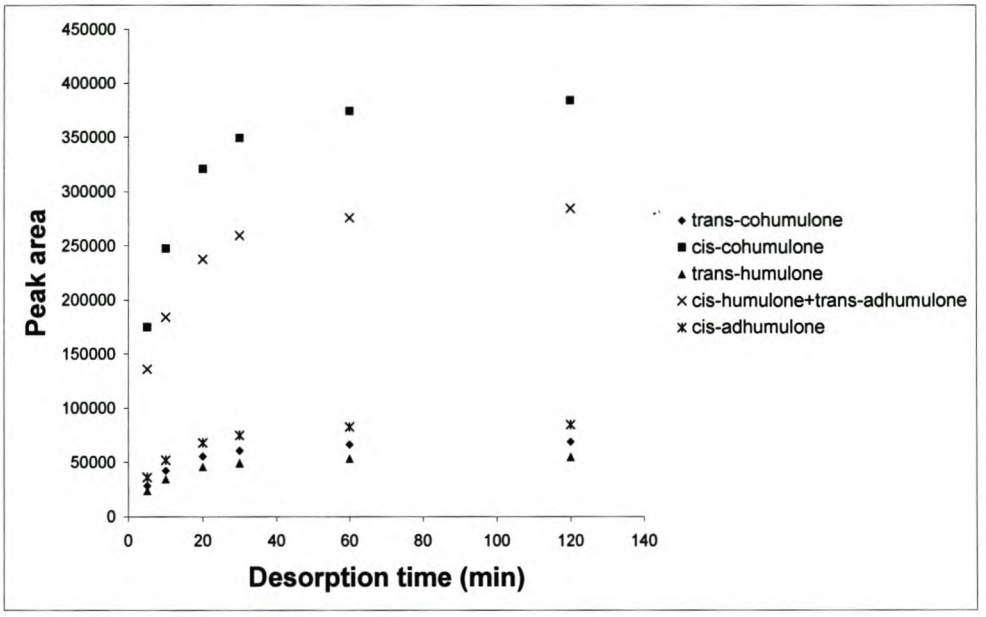

Figure 13.3: Desorption graph for beer iso-α-acids.

13.3.4 SBSE-LD-MEKC Analysis of Beer

Although iso-α-acids are generally present in sufficiently large amounts (typically 20-50 ppm) in beers, direct injections of beer followed by MEKC analysis is not feasible for the analysis of these compounds due to interference of matrix components. Performing SBSE-LD prior to injection isolates the iso-α-acids and reduced iso-α-acids from their matrix and clean extracts are obtained. A comparison between direct injection and injection after extraction is shown in figure 13.4. The MEKC method employed allows simultaneous determination of the iso-α-acids and the reduced iso-α-acids. Due to the extended light path ("bubble cell"), a S/N gain of ca. 3 is obtained compared to regular 50 μm capillaries. Additionally, the loss of efficiency due to Joule heating is minimized due to the narrow internal diameter of the capillary.

A calibration graph was constructed by injecting standard solutions of iso-α-acids in the concentration range of 23.4 to 234.0 ppm. Each level was analyzed in duplicate. Good

linearity was obtained in this concentration range. The calibration graph is depicted in figure 13.5. Reproducibility of the method was evaluated by 5 analyses of the same beer using 5 new stir bars. The results are summarised in table 13.2.

	Relative areas	
Acid	RSD(%)* migration time	RSD(%)* Relative normalised area
cis-cohumulone	0.14	5.02
trans-cohumulone	0.15	5.67
cis-adhumunlone	0.16	5.23
cis-humulone	0.23	4.41
trans-adhumulone	0.19	2.33
trans-humulone	0.26	3.08

* n = 5

Table 13.2: Repeatability of SBSE-LD-MEKC analyses.

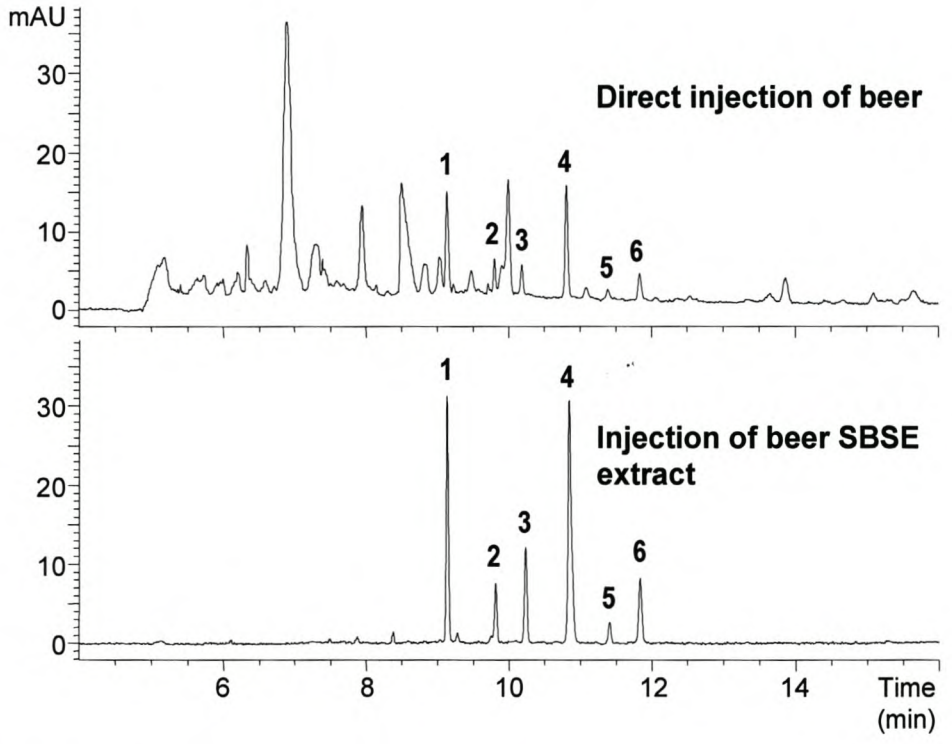

Figure 13.4: Comparison of MEKC analysis of direct injection of beer and injection after SBSE-LD. Operating parameters: see Experimental. Peak numbers: 1 = cis-iso-cohumu-lone, 2 = trans-iso-cohumulone, 3 = cis-iso-adhumulone, 4 = cis-iso-n-humulone, 5 =trans-iso-adhumulone, 6 = trans-iso-n-humulone.

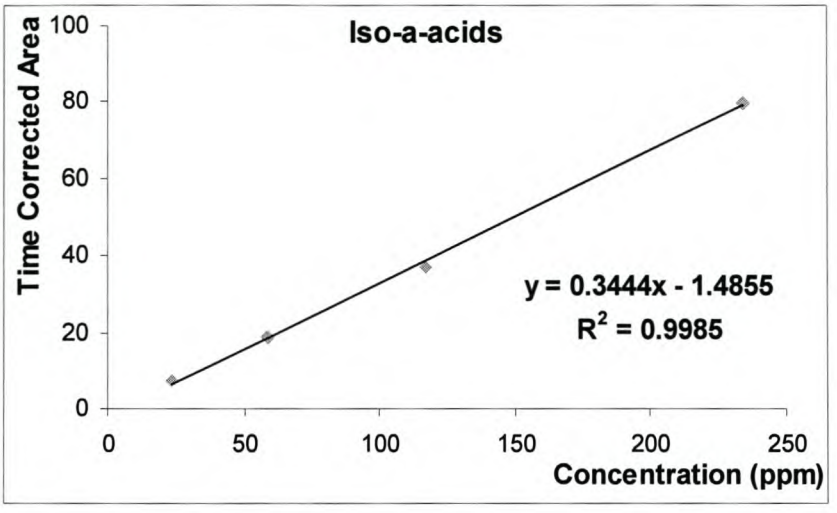

Figure 13.5: Calibration data for the iso-α-acids. The compounds were taken as one group.

13.3.5 Comparison of Various Beers

A selection of beers was analyzed using the methodology described above. Results were compared for the total iso-α-acid content and for the presence or absence of reduced iso-α-acids. These reduced forms are not allowed in German beers due to the "Reinheitsgebot", which allows only the use of natural hop compounds in the brewing process.

Representative electropherograms of extracts of beers are shown in figure 13.6. Indeed, the German beer (electropherogram 2 in figure 13.6) does not contain any reduced iso-α-acids. Another typical characteristic of this beer is the relatively low content of trans-iso-α-acid isomers present. The profile of the non-German lager (electropherogram 3 in figure 13.6) is typical for a Western European lager beer. These beers usually contain iso-α-acids to which a portion of tetrahydro-iso-α-acids is added, mainly to improve the foam stability. The procedure is more common in large breweries, compared to smaller ones who often still use only natural hop compounds. The difference between the two beers that are contained in clear bottles is striking. Because they are not protected from the light, their flavour should be stabilized in some way. For one beer this is the case, i.e. it is mainly made out of rho- and tetrahydro-iso-α-acids (electropherogram 5 in figure 13.6). Also an amount of iso-α-acids is present, however, the concentration is significantly lower compared to the other

beers. In the other beer on the other hand, only iso-α-acids are detected (electropherogram 4 in figure 13.6). No reduced iso-α-acids are present. This beer is also characterized by a relatively high content of iso-cohumulone compared to iso-ad- and iso-n-humulone.

Whereas the reduced iso-α-acids were only investigated on their presence in the beers, the iso-α-acids themselves were quantified in the various beers. For this reason, the amount of iso-α-acids in the extract of the standard solution was calculated using the constructed calibration graph. This concentration was calculated back to the initial concentration in the standard solution (23.4 ppm). The factor used for this is then applied to the SBSE extracts of the various beers. The results were compared with results obtained by HPLC after direct injection of the beers. The recovered amounts are depicted in table 13.3. The recovered amounts in MEKC are in good agreement with the actual amounts obtained by direct injection of beers by HPLC.

13.4 Concluding Remarks

Stir bar sorptive extraction followed by liquid desorption and MEKC separation is a simple, efficient and reproducible new method for the analysis of beer iso-α-acids. The procedure yields very clean extracts containing nearly only the iso-α-acids and reduced iso-α-acids. Parameters affecting both sorption and desorption were determined. The developed MEKC method is fast and enables the analysis of both iso-α-acids and reduced iso-α-acids. The quantitative results are in good agreement with the results obtained by HPLC.

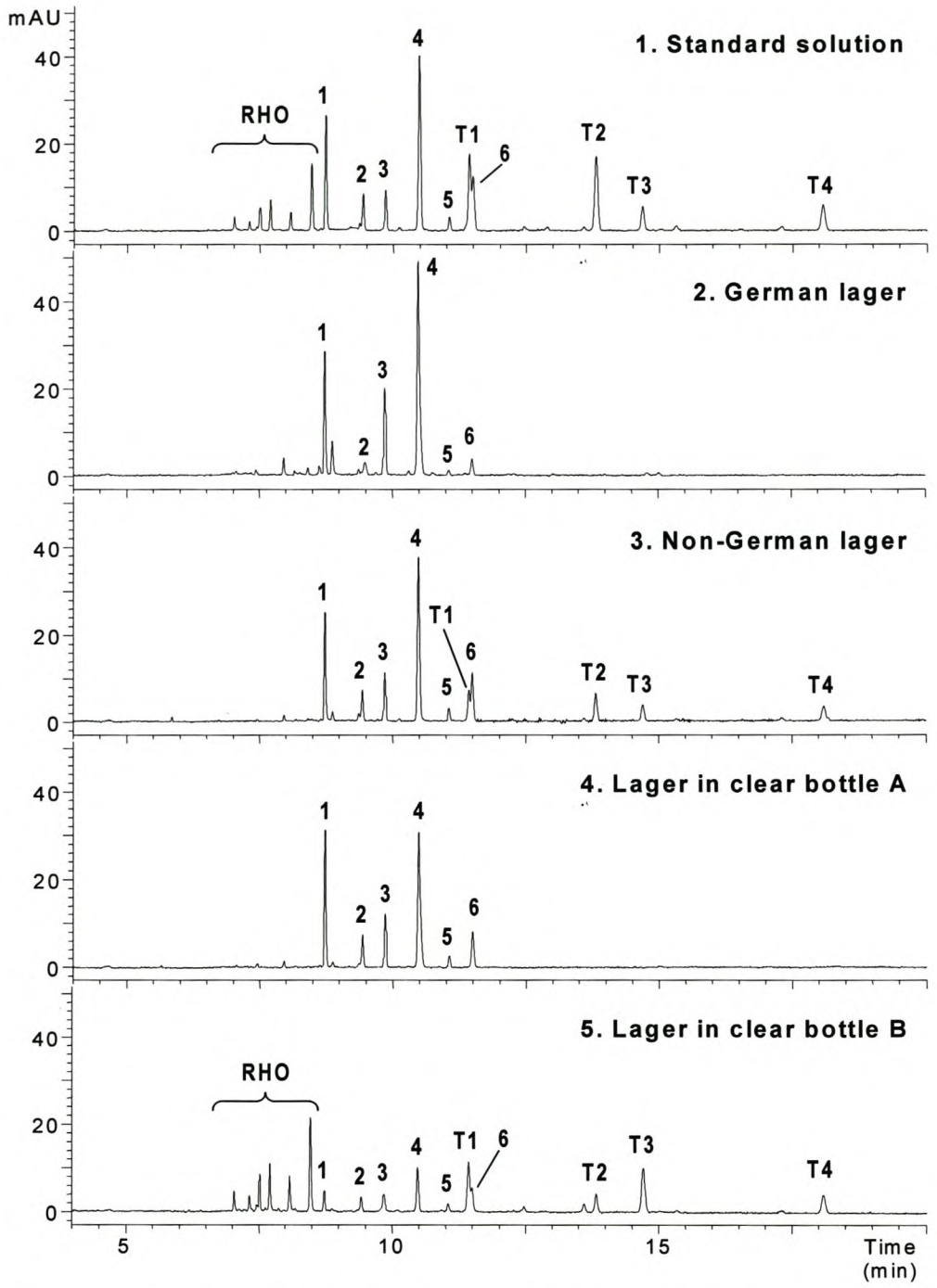

Figure 13.6: Comparison of MEKC analyses of a standard solution and various beers after SBSE-LD. Operating parameters: see Materials and Methods. Peak numbers: 1-6 = see figure 1, RHO = rho-iso-α-acids, T1 = cis-tetrahydroiso-cohumulone, T2 = trans-tetrahydroiso-co-humulone, T3 =cis-tetrahydroiso-n-humulone, T4 = trans-tetrahydroiso-n-humulone.

Sample	Concentration iso-α-acids (ppm)			Tetrahydro-iso-α-acids[a]	Rho-iso-α-acids[a]
	MEKC (extract)	MEKC (beer)	HPLC (beer)		
Standards	82.85	23.40[b]			
German lager	90.30	25.50	23.23	-	-
Non-German lager A	62.05	17.52	20.98	+	+
Non-German lager B	70.73	19.98	18.04	+	+
Non-German lager C	84.03	23.74	23.91	+	+
Lager in clear bottle A	80.11	22.63	20.47	-	-
Lager in clear bottle B	22.64	6.39	6.25	+	+
Light stable lager	0.00	0.00	0.00	+	+
Belgian trappist	90.66	25.61	24.10	-	-

a) + = present, - = not present

b) 23.40 ppm is the original concentration in the standard solution before extraction

Table 13.3: Found iso-α-acids (by MEKC and HPLC) and reduced iso-α-acids in the investigated beers.

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14

Conclusions

The goals of this study were two-fold in nature: the development of suitable methodologies for the analysis of non-volatile fractions in wines; and the interpretation of data by chemometrics for South African wines using the developed methods. In the first part of the thesis, background information is provided on various aspects of wine chemistry of interest in this and other studies, as well as on the analytical techniques employed herein.

Since this work was undertaken primarily from the point of view of analytical chemistry, the majority of the chapters deal with aspects pertinent to the development of appropriate analytical methods. These studies were complicated from the beginning by the intricate nature of the sample to be analysed, more especially so in the case of red wine. Thus, as a first step the development of a suitable sample preparation method using solid phase extraction (SPE) was undertaken (Chapter 4). This method provided samples suitable for analysis of organic acids, sugars and phenolic compounds in one procedure. Another relevant goal was the choice of separation technique. Of particular interest in the analysis of non-volatile compounds, in view of recent literature trends, is the possibilities offered by electrodriven separation techniques as alternatives to the more established high performance liquid chromatographic (HPLC) methods. This aspect was investigated in a number of chapters. In Chapter 5, the inherent properties of capillary zone electrophoresis (CZE) were exploited for the analysis of organic acids in wine without sample preparation. The method was shown to be more reliable than currently used HPLC methods, as well as recently published CZE methods. Chapter 6 deals with the evaluation of electro- and pressure driven separation methods for the analysis of sugars in dry wines. Although none of the methods investigated were ideally suited for this purpose, HPLC with refractive index (RI) or evaporative light scattering detection (ELSD) provided the best results. The lack of a sufficiently sensitive detection method in CE was at least partially to blame for the poor results; certain inherent problems when using indirect UV detection for the analysis of carbohydrates were highlighted. In Chapter 7, CE and HPLC were compared for the analysis of phenolic compounds in wine by mass spectrometric (MS) detection. LC-MS was demonstrably superior to CE-MS for these analyses, offering increased sensitivity and resolving power under conditions suitable for MS detection. Consequently, the impressive combination of LC with MS detection was further explored in the following chapters. Direct injection of wine samples followed by LC separation and diode array and

electrospray (ES)-ion trap MS detection was introduced as an exceedingly powerful method for the analysis of diverse non-coloured phenolic compounds in red wines. While quantification was preferably performed using UV detection, the coupling of MS allowed the identification of numerous non-standard compounds, including some proposed compounds reported for the first time in wines (Chapter 8). Similarly, in Chapter 9 it is shown that the use of LC-MS with positive electrospray ionisation allows the identification of forty-four anthocyanins and their derived compounds in red wines. Based on these results, a robust LC-DAD method was proposed for the routine analysis of anthocyanins. In Chapter 12, the two methods are developed as solutions to a problem from the wine industry: the identification of artificial dyes added to red wines to improve the colour. An LC method with liquid-liquid extraction was shown to be suitable for the detection of low amounts of both target dyes. Also, an elegant CE method was developed together with a SPE sample clean-up step to eliminate sample interferences. Although not directly related to the principal goals of this study, the very promising results obtained from the application of stir bar sorptive extraction (SBSE) in conjunction with liquid desorption and micellar electrokinetic chromatography (MEKC) for the analysis of hop derived bitter acids in beer is reported in Chapter 13.

In the second part of the thesis, the results obtained from the analysis of South African red and white wines using the above-described methods were evaluated by chemometric methods. A brief overview of these methods is presented in Chapter 10. In Chapter 11, the results from this study are compared to literature reports and discussed. Several pattern recognition techniques were employed to investigate the inherent properties of the data. Linear discriminant analysis (LDA) was used to obtain classification functions for both red and white wines based on their chemical composition. LDA in combination with the phenolic data showed excellent results for the classification of wines according to variety, independent from wine vintage or origin.

In conclusion, several valuable analytical methods have been developed, allowing the quantitative analysis of non-volatile fractions of South African wines. The generated data have been useful for the classification of wines according to variety, and it is hoped that future expanding of such a data set will be beneficial to the wine industry in general.

Aside from expansion of the number of analysed wines, further research should also focus on the determination of additional non-volatile compounds (amino acids, metals, biogenic amines, proteins, and possibly even wine tannins), as well as combination of non-volatile data with those obtained for the volatiles for the same wines (this is already in progress). Furthermore, combination of such a broadened chemical data-base with detailed sensory data and suitable chemometric methodologies, should allow useful conclusions to be drawn regarding these two properties of wine. Also, classification of wine samples according to different criteria (geographical origin, vintage and quality) should then be attainable.

Appendix A

Analysed Wines

Cabernet Sauvignon	Vintage	Merlot	Vintage	Pinotage	Vintage	Red Blends	Vintage
CC Cab S 88	1988	Plaisir Merlot 99	1999	IWBT Pinot 01[c]	2001	RdB 97	1997
CC Cab S 92	1992	YW 49/1[b]	2003	K'kop Pinot	1999	RdB 98	1998
CC Cab S 94	1994	YW 49/2[b]	2003	Lutzv Pinot 99	1999	RdB 99	1999
CC Cab S 95	1995	YW 49/3[b]	2003	IWBT Pinot 02[c]	2002	RdB 00	2000
CC Cab S 96	1996	YW 49/4[b]	2003	M Vilj Pinot	2001		
CC Cab S 97	1997	YW 49/5[b]	2003	WK Pinot B16/t55[a]	2001		
CC Cab S 98	1998	YW 49/6[b]	2003	KWV Pinot A06/t69[a]	2001		
Stel'ryck Cab S 98	1998	YW 49/7[b]	2003	YW 46/1[b]	2003		
Prl Cab S A06/T99[a]	2001	YW 49/8[b]	2003	YW 46/2[b]	2003		
RR Cab S B16/T22[a]	2001	YW 49/9[b]	2003	YW 46/3[b]	2003		
YW 48/1[b]	2003			YW 46/4[b]	2003		
YW 48/2[b]	2003						
YW 48/3[b]	2003						

Shiraz	Vintage	Ruby Cabernet	Vintage	Tinta Barroca	Vintage
Villiera Shiraz	2001	KWV R Cab A06/t100[a]	2001	KWV T Barr B16/t3[a]	2001
Eikdl Shiraz	2001	KWV R Cab A06/t94[a]	2001		
Boland Shiraz	1999	R Cab A06/t55[a]	2001		
Neil J Shiraz 01	2001	YW 57/1[b]	2003		
Landsk Shiraz 00	2001	YW 57/2[b]	2003		
WK Shiraz B16/52[a]	2001	YW 57/3[b]	2003		
KWV Shiraz B16/t4[a]	2001	YW 57/4[b]	2003		
WK Shiraz A06/t106[a]	2001	YW 57/5[b]	2003		
YW 55/1[b]	2003	YW 57/6[b]	2003		
YW 55/2[b]	2003	YW 57/7[b]	2003		
YW 55/3[b]	2003				

[a]Tank samples obtained from KWV

[b]Wines from the Young Wine Show 2003

[c]Obtained from the Institute for Wine Biotechnology, Stellenbosch, South Africa

Table A1: Red wine samples analysed in this study.

Chardonnay	Vintage	Sauvignon Blanc	Vintage	Chenin Blanc	Vintage
CC Chard 99	1999	R'son S Bl 02	2002	KWV Ch Bl 01	2001
CC Chard 00	2000	KWV S Bl 01	2000	YW 6/1[a]	2003
CC Chard 01	2001	KWV S Bl 00	2001	YW 6/2[a]	2003
R'son Chard 02	2002	YW 8/1[a]	2003	YW 6/3[a]	2003
KWV Chard 01	2001	YW 8/2[a]	2003	YW 6/4[a]	2003
YW 9/1[a]	2003	YW 8/3[a]	2003	YW 15/1[a]	2003
YW 9/2[a]	2003	YW 8/4[a]	2003	YW 15/2[a]	2003
YW 9/3[a]	2003	YW 8/5[a]	2003	YW 15/3[a]	2003
YW 9/4[a]	2003	YW 8/6[a]	2003	YW 15/4[a]	2003
YW 9/5[a]	2003	YW 8/7[a]	2003	YW 15/5[a]	2003
YW 9/6[a]	2003	YW 8/8[a]	2003		
YW 9/7[a]	2003	YW 8/9[a]	2003		
YW 9/8[a]	2003	YW 8/10[a]	2003		
YW 9/9[a]	2003				
YW 9/10[a]	2003				

[a]Wines from the Young Wine Show 2003

Table A2: White wine samples analysed in this study.

Appendix B

Quantitative Results

Compound	Abbrev.	Stel'ryck Cab S 98	Plaisir Merlot 99	RdB 99	RdB 97	CC Cab S 96
ORGANIC ACIDS	**ACIDS**					
tartaric	TA	1406	1914	1360	1099	1231
malic	MA	27	256	82	59	34
citric	CAc	21	0	0	0	0
succinic	SAc	863	1177	997	1015	839
acetic	AA	290	330	949	878	830
lactic	LA	3711	1907	3295	2976	3312
POLYPHENOLS	**PHENOLS**					
Gallic acid	GA	18.8	39.8	27.8	27.8	36.4
Protocatechuic acid	PrCA	2.4	2.9	4.0	6.1	4.2
Caffeoyl-tartaric acid	CTA	22.6	20.0	12.7	14.4	17.9
Catechin	C	17.2	27.9	23.8	32.4	40.5
Coumaroyl-tartaric acid	CMA	15.2	11.2	5.3	6.6	9.0
Vanillic acid	VA	6.8	6.2	3.3	4.1	2.9
caffeic acid	CA	11.9	8.0	7.1	5.4	5.6
syringic acid	SA	11.1	4.6	7.0	7.5	6.6
epicatechin	EC	7.9	20.1	12.8	15.5	19.4
p-coumaric acid	PCA	9.2	9.3	7.0	7.4	7.3
myricetin-glucoside	Mg	4.2	1.2	2.4	0.2	0.0
ferulic acid	FA	0.5	0.3	0.4	0.4	0.0
trans-polydatin	TPd	0.3	2.0	1.4	1.2	0.0
Quercetin-glucoside	Qg	16.1	13.1	11.6	7.8	6.8
Isorham/Kaempf-glucoside	IrKg	8.8	6.9	6.4	4.5	7.8
cis-polydatin	CPd	0.2	1.8	0.0	0.0	0.0
Myrecitin	My	8.6	5.9	4.8	1.2	8.6
Resveratrol	Re	1.0	3.2	1.9	1.9	1.3
Quercetin	Q	8.4	9.2	4.8	1.5	8.7
cis-resveratrol	CR	0.9	1.4	0.9	0.8	0.5
Kaempferol	Ka	2.3	2.5	0.2	0.1	0.8
Isorhamnetin	Ir	2.5	3.0	0.4	0.1	0.9
SUGARS	**SUGARS**					
fructose	F	293	622	411	256	218
glucose	G	319	640	333	371	217
ANTHOCYANINS	**ANTHOC.**					
delphinidin-3-glucoside	Dg	0.5	1.3	0.2	0.3	0.6
cyanidin-3-glucoside	Cg	0.0	0.2	0.0	0.1	0.0
petunidin-3-glucoside	Ptg	0.5	1.6	0.2	0.4	0.6
peonidin-3-glucoside	Png	0.3	1.2	0.1	0.2	0.3
malvidin-3-glucoside	Mg	4.8	9.4	2.6	3.2	6.1
delphinidin-(6-acetyl)-3-glucoside	DgAc	0.0	0.0	0.0	0.0	0.1
Vitisin A (mv-3-glucoside-pyruvic acid)	MgPA	1.6	1.2	1.1	0.9	1.1
cyanidin-(6-acetyl)-3-glucoside	CgAc	0.6	0.6	0.4	0.3	0.3
petunidin-(6-acetyl)-3-glucoside	PtgAc	0.1	0.4	0.0	0.1	0.2
delphinidin-(6-coumaroyl)-3-glucoside	DgCm	0.0	0.1	0.0	0.0	0.0
peonidin-(6-acetyl)-3-glucoside	PngAc	0.0	0.3	0.0	0.0	0.0
malvidin-(6-acetyl)-3-glucoside	MgAc	0.7	1.8	0.3	0.3	1.4
petunidin-(6-coumaroyl)-3-glucoside	PtgCm	0.0	0.1	0.0	0.0	0.0
peonidin-(6coumaroyl)-3-glucoside	PngCm	0.0	0.3	0.0	0.0	0.0
malvidin-(6-coumaroyl)-3-glucoside	MgCm	2.2	1.8	1.0	0.6	1.8
malvidin-3-glucoside-4-vinylphenol (A)	MgVp	0.3	0.5	0.4	0.1	1.5
TotalAnthocyanins	TOTA	11.7	20.6	6.1	6.7	14.0
pH	**pH**	3.55	3.49	3.54	3.45	3.61

Table B1: Quantitative results obtained for the red wines. All values reported in $mg \cdot mL^{-1}$.

Compound	CC Cab S 95	CC Cab S 94	CC Cab S 88	CC Cab S 92	IWBT Pinot 01	K'kop Pinot	Lutzv Pinot 99	IWBT Pinot 02	M Vilj Pinot
ACIDS									
TA	1057	1335	1297	1099	1354	1759	1752	1654	1320
MA	38	81	72	222	1786	92	884	52	0
CAc	0	0	0	0	207	0	118	132	63
SAc	1411	1231	958	1443	1141	641	673	627	593
AA	835	798	963	756	496	697	372	989	845
LA	2596	2299	2476	2398	841	3651	2458	2906	5398
PHENOLS									
GA	27.5	35.2	19.5	39.0	27.4	25.5	38.7	29.5	42.0
PrCA	4.4	3.5	8.0	5.6	4.4	5.2	4.1	4.4	2.9
CTA	25.0	18.6	7.4	16.7	92.6	22.8	38.1	39.3	56.6
C	22.2	24.4	10.4	17.1	38.2	34.3	30.3	41.0	39.1
CMA	12.4	9.2	4.9	9.9	26.0	5.4	9.1	6.8	13.2
VA	3.3	2.8	3.1	3.2	7.5	3.3	2.9	5.1	4.7
CA	6.2	5.6	4.1	4.9	9.5	60.2	31.2	18.7	29.7
SA	6.1	6.2	9.3	7.1	8.2	5.0	4.0	5.5	10.0
EC	11.5	4.7	0.7	4.9	18.5	11.2	14.6	27.7	25.5
PCA	5.1	5.0	7.7	5.5	3.2	18.0	10.8	5.3	9.7
Mg	1.2	8.3	0.0	2.5	35.9	26.2	23.4	5.1	1.1
FA	0.3	0.2	0.2	0.1	0.2	0.8	0.6	0.5	0.8
TPd	0.1	0.4	0.0	0.6	0.5	0.4	0.7	1.5	0.4
Qg	11.2	18.0	0.4	8.4	9.8	13.0	12.9	9.6	5.5
IrKg	6.7	6.6	3.7	5.5	15.5	13.4	13.6	9.6	8.8
CPd	0.2	0.0	0.0	0.0	0.4	0.0	0.5	1.7	0.0
My	4.7	0.6	0.2	0.7	1.8	4.9	1.2	4.5	8.1
Re	0.5	0.4	0.1	0.4	0.6	0.5	0.4	1.3	1.0
Q	5.9	2.6	0.5	2.5	2.7	6.6	3.0	1.9	5.5
CR	0.4	0.3	0.0	0.2	0.3	0.4	0.2	1.0	0.9
Ka	0.6	0.7	0.1	0.4	0.5	1.1	0.8	0.1	0.6
Ir	0.7	0.4	0.1	0.4	0.3	0.6	0.1	0.4	0.6
SUGARS									
F	149	227	486	475	1955	697	764	634	1675
G	283	300	381	539	549	1438	559	1346	700
ANTHOC.									
Dg	0.1	0.0	0.0	0.1	15.0	1.1	1.2	12.3	1.6
Cg	0.0	0.0	0.0	0.0	1.2	0.1	0.0	1.3	0.2
Ptg	0.0	0.0	0.0	0.0	18.8	1.7	1.7	16.9	2.9
Png	0.0	0.0	0.0	0.0	9.0	1.0	0.8	10.8	1.6
Mg	1.0	0.7	0.6	0.5	110.1	10.3	13.1	114.8	26.1
DgAc	0.0	0.0	0.0	0.0	4.2	0.2	0.2	3.4	0.6
MgPA	1.6	1.1	0.3	1.0	6.9	1.9	1.8	3.1	3.0
CgAc	0.4	0.2	0.0	0.1	2.5	0.4	0.3	1.1	1.0
PtgAc	0.0	0.0	0.0	0.0	5.3	0.4	0.4	4.5	0.6
DgCm	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.8	0.0
PngAc	0.0	0.0	0.0	0.0	3.3	0.1	0.1	4.6	0.4
MgAc	0.0	0.0	0.0	0.0	28.4	1.9	2.1	30.6	5.7
PtgCm	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.9	0.1
PngCm	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.8	0.1
MgCm	0.7	0.7	1.1	0.5	8.6	8.3	6.5	10.7	6.4
MgVp	0.8	0.3	1.2	0.3	0.4	0.8	0.6	0.2	0.6
TOTA	4.7	3.2	3.2	2.5	216.3	28.3	28.9	217.8	50.9
pH	3.60	3.49	3.23	3.42	3.76	3.68	3.59	3.56	3.95

Table B1(continued): Quantitative results obtained for the red wines.

Compound	Villiera Shiraz	Eikdl Shiraz	Boland Shiraz	Neil J Shiraz 01	Landsk Shiraz 00	WK Shiraz B16/T52	WK Pinot B16/T55	KWV Shiraz B16/T4	KWV Pinot A06/T69
ACIDS									
TA	1351	1630	1728	1967	2645	1182	2126	1517	1288
MA	163	84	100	89	75	54	36	0	55
CAc	0	0	47	0	0	0	0	0	0
SAc	1401	949	900	964	1025	1290	720	1028	933
AA	491	747	526	610	449	718	675	839	531
LA	3507	2477	1870	2185	1636	2898	4435	4033	3498
PHENOLS									
GA	37.4	37.3	31.2	46.7	37.9	58.1	21.0	44.9	55.2
PrCA	3.8	4.1	3.8	5.2	8.7	5.0	2.0	4.3	4.5
CTA	39.6	19.9	11.0	39.6	17.3	22.9	44.8	23.7	32.6
C	48.4	39.4	30.8	60.0	39.1	49.5	23.4	41.4	52.4
CMA	17.0	10.6	5.6	16.8	8.5	10.0	10.7	13.0	6.4
VA	4.5	4.1	3.7	5.2	6.4	6.0	2.2	4.7	4.0
CA	5.5	17.8	22.0	10.1	18.0	5.4	25.1	13.2	78.6
SA	3.1	4.4	5.0	5.8	8.3	8.4	4.7	5.4	4.6
EC	26.4	30.2	14.8	34.9	9.5	28.9	10.4	26.6	31.4
PCA	3.9	7.6	15.4	4.0	12.5	5.5	9.7	7.3	19.6
Mg	29.2	44.9	15.2	21.6	18.4	14.0	0.0	21.8	23.7
FA	0.1	0.3	0.4	0.3	0.4	0.2	0.6	0.3	0.9
TPd	2.0	1.2	2.5	2.7	2.6	0.8	0.5	0.7	0.8
Qg	34.9	29.4	21.4	31.7	38.1	23.1	4.1	21.5	10.2
IrKg	24.5	33.7	14.5	13.6	19.0	20.0	7.3	20.8	11.1
CPd	2.0	1.0	1.8	2.8	1.6	0.7	0.6	0.7	0.8
My	2.4	2.6	4.0	7.9	11.6	6.6	4.5	7.8	9.6
Re	1.5	0.5	1.4	1.7	1.1	0.6	1.3	0.8	1.3
Q	15.9	9.5	10.9	20.1	19.3	19.1	5.3	18.6	10.8
CR	1.0	0.4	0.8	1.5	0.5	0.0	1.0	0.6	0.4
Ka	2.2	2.3	1.2	2.5	1.0	1.7	0.5	1.8	2.2
Ir	3.6	1.6	1.9	3.8	4.7	6.2	0.4	5.2	1.1
SUGARS									
F	473	606	526	499	647	529	263	669	528
G	727	823	842	760	1189	806	406	349	270
ANTHOC.									
Dg	9.4	3.9	0.5	7.6	1.0	0.6	0.4	0.4	12.8
Cg	0.8	0.3	0.0	1.0	0.1	0.1	0.0	0.0	0.6
Ptg	13.0	6.3	0.8	7.5	2.2	2.3	0.7	0.8	15.9
Png	10.0	3.4	1.0	5.1	2.2	2.5	0.3	0.7	6.6
Mg	72.3	39.6	6.6	39.7	21.3	21.7	7.8	8.0	93.3
DgAc	2.2	0.9	0.0	1.8	0.0	0.0	0.0	0.0	2.9
MgPA	2.1	1.9	1.7	2.0	2.2	1.8	1.7	0.9	1.6
CgAc	0.8	0.6	0.3	0.7	0.5	1.1	0.7	0.8	1.7
PtgAc	3.1	1.5	0.1	1.9	0.3	0.5	0.1	0.2	3.7
DgCm	1.2	0.5	0.0	0.4	0.1	0.1	0.0	0.0	0.6
PngAc	4.7	1.7	0.1	1.7	0.3	0.8	0.1	0.2	2.8
MgAc	21.4	11.8	0.8	11.4	3.4	6.3	1.7	2.1	22.5
PtgCm	1.5	0.7	0.0	0.5	0.2	0.1	0.0	0.0	0.7
PngCm	3.7	1.1	0.1	0.8	0.4	0.6	0.0	0.2	1.1
MgCm	9.4	6.2	1.8	5.5	3.8	4.1	5.3	1.7	11.2
MgVp	0.8	0.6	0.2	0.4	0.2	0.6	0.8	0.7	0.7
TOTA	156.5	80.9	14.1	88.0	38.4	43.2	19.7	16.7	179.0
pH	3.63	3.57	3.42	3.67	3.33	3.57	3.54	3.65	3.81

Table B1(continued): Quantitative results obtained for the red wines.

Compound	Prl Cab S A06/T99	KWV T Barr B16/T3	RR Cab S B16/T22	KWV R Cab a06/t100	KWV R Cab a06/t194	R Cab A06/t55	WK Shiraz A06/T106	YW 49/1	YW 49/2
ACIDS									
TA	690	1375	1166	0	961	1074	1308	2146	2067
MA	0	69	0	0	0	0	0	0	0
CAc	0	0	0	0	0	0	0	0	0
SAc	850	1192	1040	1293	866	1203	673	1168	1327
AA	991	898	1063	1313	739	1893	3219	574	336
LA	5401	3048	4947	6087	6055	4592	6596	1340	1824
PHENOLS									
GA	9.1	39.4	41.1	10.3	16.0	26.0	18.2	28.3	40.4
PrCA	1.1	4.0	3.1	3.3	3.2	3.9	2.2	5.7	5.8
CTA	10.8	69.8	10.4	0.3	14.6	8.6	31.7	8.7	1.9
C	28.5	33.8	57.5	11.3	35.4	38.2	26.1	54.7	68.8
CMA	3.6	30.3	3.4	0.0	6.0	3.9	10.4	3.9	3.6
VA	3.0	4.7	5.0	3.2	3.7	5.6	3.4	0.0	4.8
CA	4.5	10.7	10.4	0.0	4.6	3.9	7.9	42.9	21.6
SA	8.3	5.7	10.9	6.9	10.8	15.6	5.7	4.5	5.0
EC	14.5	5.0	29.4	2.4	26.1	26.4	14.5	37.0	57.5
PCA	5.2	6.3	7.6	0.0	9.8	6.2	7.5	11.8	4.6
Mg	0.0	1.3	3.2	26.4	9.3	2.6	0.0	29.1	30.1
FA	0.2	0.3	0.7	0.0	0.5	0.6	0.3	0.8	1.2
TPd	0.0	3.8	0.2	0.0	0.0	0.0	0.0	2.3	3.1
Qg	4.1	9.3	15.9	17.0	11.4	14.3	11.4	49.6	34.0
IrKg	6.2	10.2	11.8	13.0	7.4	10.1	7.9	16.5	14.7
CPd	0.0	3.6	0.0	0.3	0.5	0.0	0.0	1.5	2.1
My	2.7	4.6	8.6	5.5	7.3	13.9	8.0	4.4	2.7
Re	0.4	2.7	0.7	0.6	1.0	0.6	1.5	1.1	1.8
Q	3.8	8.7	9.8	8.6	8.6	10.0	14.4	17.6	11.2
CR	0.6	2.2	0.7	0.5	1.8	0.4	1.2	0.9	1.7
Ka	0.3	1.4	0.9	1.5	1.1	1.3	0.7	3.3	2.1
Ir	0.4	1.8	1.3	0.7	0.5	0.8	2.3	1.4	0.9
SUGARS									
F	95.7	129.9	277	0	0	174	80	335	408
G	115.6	72.1	343	0	162.0	152	125	406	521
ANTHOC.									
Dg	0.5	0.6	1.5	3.6	1.3	2.8	0.4	13.9	15.7
Cg	0.0	0.0	0.0	0.2	0.0	0.1	0.0	1.5	1.0
Ptg	0.8	1.4	1.5	4.6	1.8	2.8	0.6	15.6	21.3
Png	0.6	1.1	1.2	2.1	0.4	0.9	0.4	9.3	13.7
Mg	24.8	16.0	18.3	37.2	16.4	22.3	5.1	92.6	171.0
DgAc	0.1	0.0	0.0	0.4	0.4	0.7	0.0	2.0	5.8
MgPA	0.8	1.6	2.1	1.6	2.4	4.6	0.4	1.6	1.9
CgAc	0.5	0.5	1.4	1.0	0.9	1.6	0.3	0.8	0.5
PtgAc	0.2	0.2	0.3	0.4	0.1	0.3	0.0	4.5	6.5
DgCm	0.0	0.0	0.0	0.1	0.0	0.2	0.0	1.3	1.8
PngAc	0.2	0.1	0.2	0.1	0.0	0.3	0.1	3.7	8.6
MgAc	6.0	1.6	6.0	2.5	2.2	3.9	1.1	29.1	64.3
PtgCm	0.0	0.1	0.0	0.1	0.0	0.1	0.0	1.6	2.3
PngCm	0.0	0.2	0.0	0.0	0.0	0.1	0.0	2.4	4.5
MgCm	2.0	2.5	2.1	7.7	2.3	2.4	2.9	14.2	27.5
MgVp	0.7	0.7	0.6	0.8	2.7	0.4	3.3	0.2	0.3
TOTA	37.1	26.7	35.3	62.5	31.0	43.5	14.5	194.3	346.8
pH	3.90	3.62	3.57	4.22	3.82	3.64	3.62	3.62	3.74

Table B1(continued): Quantitative results obtained for the red wines.

Compound	YW 49/3	YW 49/4	YW 49/5	YW 49/6	YW 49/7	YW 49/8	YW 49/9	YW 57/1	YW 57/2
ACIDS									
TA	2599	2256	3599	2578	2431	1438	2138	2086	1954
MA	55	95	34	53	35	0	1651	82	0
CAc	196	0	92	0	57	0	137	59	128
SAc	835	1171	1126	1296	997	1108	1390	830	866
AA	630	373	363	433	407	443	240	560	400
LA	1376	1909	1101	1405	1776	1212	610	3168	2727
PHENOLS									
GA	22.0	27.0	35.0	21.8	33.3	29.0	13.9	20.1	27.4
PrCA	3.3	2.9	4.1	5.1	8.7	3.9	4.1	4.4	4.2
CTA	21.2	17.8	49.0	1.4	22.9	4.5	39.7	0.5	0.9
C	34.2	50.2	60.8	50.2	79.0	83.3	66.2	31.6	35.2
CMA	5.2	6.4	14.1	0.4	5.2	1.2	3.8	4.7	2.5
VA	3.2	3.2	3.6	5.8	5.9	5.8	3.6	0.0	5.1
CA	9.0	13.6	2.7	34.0	6.1	31.5	4.5	17.0	10.6
SA	4.2	5.3	3.3	4.0	4.4	4.0	3.4	6.3	9.9
EC	26.4	31.6	40.1	29.2	40.9	63.2	29.4	29.1	46.9
PCA	4.4	4.7	2.1	9.8	0.3	9.3	1.7	12.6	7.4
Mg	9.0	24.2	11.1	9.9	50.1	9.2	46.9	17.5	10.9
FA	0.5	0.5	0.1	1.0	0.0	0.6	0.1	1.3	1.3
TPd	3.0	1.5	4.4	1.9	2.8	7.2	1.4	1.0	0.6
Qg	21.1	28.7	39.0	23.0	57.0	17.1	52.5	28.6	22.4
IrKg	8.5	11.5	6.6	7.2	21.3	6.9	16.6	11.1	12.5
CPd	2.7	1.2	5.1	2.4	2.9	7.2	1.8	1.2	0.9
My	4.2	2.0	13.1	10.1	3.5	4.7	3.7	10.5	10.9
Re	1.8	1.7	2.3	2.1	1.0	5.8	0.7	1.9	0.9
Q	8.5	6.9	24.7	20.1	23.7	12.5	14.8	11.3	8.8
CR	1.3	1.4	2.0	2.2	1.2	3.3	0.8	1.4	1.1
Ka	1.0	1.3	2.9	2.5	5.4	1.1	3.0	1.8	1.2
Ir	0.9	0.5	2.1	1.8	1.5	1.3	1.1	0.7	1.1
SUGARS									
F	317	242	363	425	307	434	364	1738	342
G	373	220	268	307	256	417	305	312	510
ANTHOC.									
Dg	18.8	7.8	41.1	48.0	22.3	16.5	16.3	23.1	24.8
Cg	1.8	0.2	6.1	10.8	2.8	1.4	1.0	0.6	0.6
Ptg	21.8	13.8	35.8	43.3	23.1	21.3	20.8	28.3	32.2
Png	20.6	4.4	27.0	35.8	14.8	14.5	9.6	8.9	9.6
Mg	152.5	130.2	146.3	182.0	116.5	132.4	123.0	240.5	263.9
DgAc	4.5	3.2	7.3	8.8	5.6	5.4	5.2	5.1	6.9
MgPA	1.9	1.3	4.2	2.7	1.9	2.9	3.3	1.6	2.8
CgAc	1.4	0.5	1.8	1.2	0.6	1.2	1.6	0.7	1.8
PtgAc	6.3	4.0	8.8	9.5	6.5	6.4	6.1	5.0	6.6
DgCm	1.8	1.7	2.8	2.4	1.5	1.3	1.7	5.2	3.7
PngAc	8.2	4.7	7.5	9.3	4.7	8.5	5.7	3.1	4.4
MgAc	49.8	47.3	33.1	43.2	33.3	38.1	38.1	55.3	60.2
PtgCm	2.4	2.3	3.2	2.6	1.9	1.7	2.1	4.8	3.9
PngCm	5.3	2.6	4.3	4.1	2.5	5.4	2.8	2.8	3.0
MgCm	25.5	23.9	16.7	18.1	15.4	19.8	19.4	37.0	35.5
MgVp	0.4	0.0	0.2	0.4	0.6	0.2	0.2	0.6	1.0
TOTA	323.1	247.9	346.1	422.3	254.0	277.2	256.9	422.7	460.8
pH	3.35	3.34	3.30	3.53	3.55	3.54	3.54	3.65	3.85

Table B1(continued): Quantitative results obtained for the red wines.

Compound	YW 57/3	YW 57/4	YW 57/5	YW 57/6	YW 57/7	YW 48/1	YW 48/2	YW 48/3	YW 46/1
ACIDS									
TA	1824	1764	1911	1308	1630	1434	1592	1610	1832
MA	104	0	0	53	24	0	0	0	0
CAc	0	0	0	0	0	0	0	0	0
SAc	1108	972	837	987	862	1594	1237	1319	788
AA	530	489	507	586	532	739	420	556	582
LA	2970	2571	2146	2442	2027	2948	2365	1877	3122
PHENOLS									
GA	23.0	26.5	15.9	16.2	25.0	13.1	32.9	70.7	10.4
PrCA	2.3	2.8	2.5	2.5	3.1	2.1	5.9	8.1	1.2
CTA	12.0	13.6	17.3	1.2	12.5	20.1	5.5	1.3	68.5
C	40.1	33.8	39.0	24.5	28.4	21.9	53.3	93.5	10.9
CMA	4.9	6.3	8.3	2.9	6.3	6.4	1.6	1.4	17.0
VA	4.0	6.1	2.4	2.0	4.0	4.4	6.8	6.8	2.9
CA	14.7	2.8	2.0	13.6	7.8	3.0	28.6	11.3	1.9
SA	4.3	7.6	6.2	7.2	6.5	3.3	7.0	12.3	3.2
EC	21.2	36.0	33.1	24.3	20.6	11.6	29.1	47.3	1.9
PCA	8.3	6.4	3.2	9.8	6.8	1.2	6.4	2.7	1.0
Mg	27.0	7.5	33.4	19.5	0.0	22.8	34.0	6.1	20.0
FA	0.6	0.4	0.2	1.2	0.4	0.0	0.8	0.9	0.0
TPd	0.7	0.8	0.1	0.8	1.0	0.0	1.1	0.4	0.4
Qg	12.5	23.6	24.4	16.0	11.2	28.1	27.8	25.5	14.6
IrKg	8.7	11.9	10.7	9.6	8.3	12.9	15.4	17.2	6.2
CPd	0.5	1.1	0.1	1.1	1.3	0.2	0.8	0.3	0.2
My	3.6	12.4	1.8	4.9	9.1	2.0	6.5	7.1	1.7
Re	1.0	2.3	0.3	0.7	2.9	0.3	0.8	0.4	0.3
Q	5.2	11.3	5.3	4.7	8.7	10.6	13.3	9.8	3.7
CR	0.8	2.1	0.0	1.1	2.6	0.1	0.0	0.0	0.3
Ka	0.7	1.5	1.1	0.8	0.8	2.4	2.6	1.4	1.1
Ir	0.3	1.0	0.1	0.5	0.6	1.0	1.1	2.1	0.2
SUGARS									
F	308	421	511	440	289	324	332	510	752
G	316	549	322	510	361	252	297	535	205
ANTHOC.									
Dg	26.2	19.8	5.8	19.4	17.0	5.5	11.1	6.9	7.6
Cg	1.2	0.7	0.1	0.4	0.5	0.2	0.5	0.4	0.2
Ptg	25.6	24.1	10.9	26.8	21.7	7.0	15.6	8.0	15.2
Png	10.1	10.6	2.0	6.3	7.3	5.0	6.6	5.3	4.4
Mg	194.8	197.0	116.2	257.6	195.8	122.4	167.1	97.4	175.4
DgAc	8.2	4.5	2.3	4.9	4.4	2.3	3.3	1.5	4.0
MgPA	1.8	2.4	1.0	1.4	1.3	1.5	2.5	3.1	1.2
CgAc	1.0	1.3	0.5	0.7	0.6	1.3	1.8	1.1	0.8
PtgAc	6.5	4.6	3.0	4.9	4.4	3.0	4.3	1.0	5.9
DgCm	1.1	2.6	2.3	3.2	2.7	0.3	1.1	0.3	1.0
PngAc	4.7	3.8	1.0	2.8	3.3	4.1	3.4	2.0	4.7
MgAc	59.9	44.0	33.7	52.4	47.2	66.5	63.8	38.1	64.0
PtgCm	1.1	2.8	2.7	3.0	2.9	0.4	1.3	0.2	1.8
PngCm	1.2	2.1	0.8	1.9	2.2	1.1	1.2	0.3	1.7
MgCm	14.0	29.4	28.2	23.4	26.8	16.6	21.6	7.3	26.2
MgVp	2.6	0.6	1.4	0.7	0.2	1.1	1.2	0.3	0.2
TOTA	359.9	350.4	211.8	409.8	338.3	238.4	306.4	173.3	314.4
pH	3.83	4.06	3.60	3.82	3.74	3.89	3.84	3.82	3.90

Table B1(continued): Quantitative results obtained for the red wines.

Compound	YW 46/2	YW 46/3	YW 46/4	YW 55/1	YW 55/2	YW 55/3	CC Cab S 97	CC Cab S 98	RdB 98	RdB 00
ACIDS										
TA	1552	2434	2113	1220	1737	1225	1534	1342	1196	1357
MA	0	0	0	0	178	0	70	41	39	80
CAc	0	78	146	0	132	0	0	0	0	0
SAc	730	796	569	1340	541	1133	1187	1090	967	900
AA	470	386	535	676	430	851	669	973	820	855
LA	2462	1812	2131	3222	2932	1661	3049	3180	3742	2636
PHENOLS										
GA	19.4	30.1	17.6	23.3	35.7	33.0	38.8	38.7	33.5	38.4
PrCA	2.4	3.7	1.0	3.1	3.7	3.5	7.3	5.9	7.3	7.4
CTA	11.5	47.8	49.2	19.1	31.9	0.6	16.6	32.8	17.5	24.0
C	38.0	38.9	25.9	39.9	64.2	31.2	28.6	36.7	30.8	37.6
CMA	2.2	9.0	10.7	9.0	14.2	0.4	9.1	16.0	8.1	9.5
VA	0.0	4.8	4.4	7.9	11.2	7.3	2.1	2.1	0.0	0.0
CA	80.0	21.1	7.7	12.6	9.9	29.2	5.3	6.6	6.9	10.0
SA	4.0	3.9	3.9	5.3	7.2	5.8	6.6	7.1	5.3	6.3
EC	27.8	18.5	17.0	34.7	58.3	24.0	18.3	22.9	23.8	21.4
PCA	10.7	2.2	1.6	4.1	8.3	6.5	7.8	3.5	9.1	8.0
Mg	35.8	23.9	7.5	18.3	0.0	31.9	0.9	4.0	1.8	2.2
FA	0.9	0.2	0.1	0.2	0.3	1.0	0.3	1.2	0.3	0.4
TPd	0.7	0.8	1.0	0.3	1.6	1.7	0.4	0.4	0.8	1.3
Qg	23.8	18.0	9.0	15.9	10.1	32.6	1.7	18.4	12.4	13.6
IrKg	12.6	11.1	10.4	15.4	13.4	34.5	5.9	8.3	5.7	7.9
CPd	0.3	0.3	0.9	1.6	2.0	1.2	0.0	1.1	0.0	0.0
My	1.5	1.5	4.9	2.4	10.1	2.6	6.3	9.4	6.9	7.8
Re	0.5	0.3	0.9	1.3	2.9	0.6	1.9	0.7	1.5	1.5
Q	4.1	6.3	4.0	7.3	14.3	15.2	8.1	12.2	6.2	10.3
CR	0.2	0.1	0.5	1.1	2.8	0.2	0.8	0.5	0.6	0.4
Ka	1.1	2.2	0.5	0.6	1.7	2.2	0.4	1.3	0.2	0.7
Ir	0.3	0.5	0.6	2.0	4.3	3.0	0.5	1.2	0.4	1.3
SUGARS										
F	490	522	746	387	772	564	418	309	265	757
G	525	695	419	238	443	442	473	292	271	333
ANTHOC.										
Dg	14.7	7.2	11.0	6.5	6.2	5.5	0.38	0.49	0.69	1.19
Cg	0.6	0.3	0.7	0.4	0.4	0.4	0.00	0.00	0.00	0.06
Ptg	23.0	13.4	23.4	14.9	15.3	12.3	0.42	0.62	0.80	1.51
Png	9.0	5.4	8.8	11.4	10.1	10.5	0.46	0.63	0.56	1.23
Mg	205.3	149.8	194.0	151.3	146.0	134.7	4.35	6.42	7.56	13.94
DgAc	4.8	2.0	4.5	2.5	2.2	1.9	0.10	0.08	0.07	0.22
MgPA	1.4	1.2	2.5	1.3	4.2	3.2	0.96	1.18	0.76	1.35
CgAc	0.2	0.9	1.6	0.7	1.9	1.9	0.24	0.22	0.24	0.29
PtgAc	6.5	3.9	7.5	4.3	5.0	3.8	0.13	0.34	0.14	0.36
DgCm	1.6	0.8	1.1	0.9	0.9	1.6	0.00	0.00	0.00	0.00
PngAc	6.3	3.8	7.5	7.6	6.7	9.1	0.00	0.00	0.00	0.12
MgAc	71.3	52.7	62.2	46.1	38.6	60.8	0.36	1.15	0.96	2.39
PtgCm	3.0	1.6	2.3	1.7	1.7	3.6	0.00	0.00	0.00	0.00
PngCm	3.3	1.7	2.6	4.4	4.8	7.4	0.00	0.00	0.00	0.13
MgCm	34.8	22.7	25.6	16.3	23.3	33.4	0.66	0.68	1.01	1.76
MgVp	0.1	0.3	0.1	0.2	0.9	0.1	0.56	0.13	0.20	0.16
TOTA	385.9	267.7	355.6	270.5	268.1	290.2	8.6	11.9	13.0	24.7
pH	3.74	3.39	3.58	3.95	3.84	3.79	3.41	3.71	3.65	3.49

Table B1(continued): Quantitative results obtained for the red wines.

Compound	Rob S Bl 02	R'son Chard 02	KWV S Bl 00	KWV S Bl 01	KWV Ch Bl 01	KWV Chard 01	YW 8/1	YW 8/2
ACIDS								
TA	1830	1280	1138	1307	933	938	1627	2048
MA	3047	2170	2814	2538	2902	2381	2416	3260
CAc	323	233	374	269	214	141	259	245
SAc	407	484	312	356	159	383	203	448
AA	651	401	327	296	383	287	207	112
LA	612	744	318	216	320	987	150	203
PHENOLS								
GA	0.8	0.8	5.6	1.1	1.2	2.3	0.5	0.6
PrCA	1.8	1.9	1.4	1.4	1.3	1.8	1.2	1.1
CTA	3.6	3.4	16.8	6.9	13.5	10.4	5.7	20.5
C	2.9	3.6	11.4	6.2	5.3	7.7	3.8	5.5
CMA	1.9	0.5	4.5	1.3	2.7	1.3	0.0	5.6
VA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CA	2.0	2.8	7.1	5.5	1.7	4.7	2.0	0.4
SA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EC	1.1	1.3	5.6	2.3	0.0	7.8	1.4	1.7
PCA	1.1	1.8	5.0	2.9	0.5	2.0	0.8	0.2
Mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FA	0.6	0.7	0.3	0.5	0.3	0.5	0.4	0.3
TPd	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Qg	0.0	0.8	0.0	0.0	0.0	1.0	0.6	0.0
IrKg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CPd	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1
My	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Re	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
Q	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
CR	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ka	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ir	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUGARS								
F	478	1712	1605	1562	3318	1319.5	667	1855
G	488	1686	1626	1641	2859	588.2	555	1763
pH	3.27	3.49	3.45	3.41	3.47	3.48	3.50	3.29

Table B2: Quantitative results obtained for the white wines. All values reported in $mg \cdot mL^{-1}$.

Compound	YW 8/3	YW 8/4	YW 8/5	YW 8/6	YW 8/7	YW 8/8	YW 8/9	YW 8/10	YW 9/1
ACIDS									
TA	1994	1541	2395	1847	1998	2777	2132	3256	1523
MA	2458	2772	3016	2936	2124	2144	2976	0	0
CAc	185	217	278	243	195	294	265	0	166
SAc	275	235	194	175	235	193	278	702	546
AA	397	269	199	271	624	389	429	183	397
LA	156	129	172	232	192	281	235	1406	3116
PHENOLS									
GA	2.1	10.5	1.7	1.8	0.8	3.6	0.7	1.2	0.9
PrCA	1.3	0.8	0.8	0.9	1.3	1.4	1.2	1.1	1.5
CTA	9.6	29.1	22.2	20.9	11.8	10.2	27.9	0.3	1.6
C	14.9	22.3	13.0	16.2	5.6	7.3	9.4	3.3	6.0
CMA	1.9	5.5	6.3	3.0	4.3	0.6	9.2	0.0	0.2
VA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CA	3.4	1.3	1.4	6.7	1.2	10.0	0.5	0.4	0.6
SA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EC	9.7	19.2	10.1	11.7	2.6	4.7	5.2	0.7	1.1
PCA	2.1	0.4	0.4	3.3	1.1	3.6	0.5	0.0	0.1
Mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FA	0.4	0.0	0.2	0.4	0.6	0.8	0.3	0.0	0.2
TPd	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Qg	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9
IrKg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CPd	0.3	0.0	0.1	0.0	0.3	0.0	0.0	0.0	0.1
My	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Re	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.0	0.0
Q	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
CR	0.0	0.0	0.6	0.0	0.1	0.0	0.0	0.0	0.1
Ka	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ir	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUGARS									
F	1633	4107	1565	1046	563	719	1061	245	176
G	596	284	1626	860	453	613	1141	180	145
pH	3.21	3.30	3.32	3.40	3.48	3.39	3.20	3.25	3.91

Table B2(continued): Quantitative results obtained for the white wines.

Compound	YW 9/2	YW 9/3	YW 9/4	YW 9/5	YW 9/6	YW 9/7	YW 9/8	YW 9/9	YW 9/10
ACIDS									
TA	2247	1975	2277	1691	3159	2118	921	1451	1115
MA	2329	2329	2116	2678	2055	441	3178	2305	2380
CAc	198	159	153	203	297	141	220	226	177
SAc	226	416	206	192	365	421	453	311	380
AA	390	264	308	352	178	308	95	380	44
LA	237	655	340	402	200	2120	169	335	180
PHENOLS									
GA	1.0	1.0	3.7	1.3	1.5	1.1	0.8	2.2	2.5
PrCA	1.8	1.1	1.7	1.1	1.9	1.3	1.0	2.2	1.5
CTA	3.9	18.1	17.4	12.2	2.1	1.7	13.2	17.7	4.8
C	7.0	8.0	16.2	8.2	6.9	3.6	6.6	8.1	15.2
CMA	0.5	2.9	3.3	1.1	0.0	2.1	0.4	2.7	0.1
VA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CA	2.4	2.2	1.1	3.1	5.2	1.1	9.1	3.3	4.6
SA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EC	1.7	5.6	16.8	8.5	3.4	1.8	3.9	6.7	16.5
PCA	0.9	0.9	1.9	1.7	3.3	0.0	2.2	1.0	0.7
Mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FA	0.3	0.3	0.4	0.5	0.8	0.2	0.3	0.2	0.6
TPd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Qg	1.7	0.4	0.0	0.0	0.5	1.5	0.0	0.0	0.9
IrKg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CPd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
My	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Re	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.3	0.1
Q	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CR	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Ka	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ir	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUGARS									
F	2654	387	1317	2123	657	274	8499	1313	302
G	822	383	1056	488	622	329	440	576	300
pH	3.42	3.49	3.36	3.57	3.21	3.58	3.59	3.57	3.94

Table B2(continued): Quantitative results obtained for the white wines.

Compound	YW 6/1	YW 6/2	YW 6/3	YW 6/4	YW 15/1	YW 15/2	YW 15/3	YW 15/4	YW 15/5
ACIDS									
TA	2547	1370	1943	2086	3037	1916	2205	2713	1526
MA	2740	2594	2367	2403	1568	2041	2491	1554	3491
CAc	223	298	269	251	344	260	314	275	254
SAc	170	388	314	409	435	629	232	328	546
AA	360	406	371	202	831	182	195	492	358
LA	152	157	309	363	169	349	298	124	216
PHENOLS									
GA	1.1	3.5	1.1	1.3	5.0	1.6	29.3	1.7	1.3
PrCA	1.1	1.5	0.8	1.0	1.4	0.9	1.6	0.9	1.5
CTA	2.4	2.7	13.8	14.6	19.7	15.7	6.7	15.1	16.2
C	4.1	7.1	4.4	5.3	10.0	4.3	3.9	4.8	4.5
CMA	0.3	0.5	2.2	2.7	1.7	1.6	1.6	2.6	1.7
VA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CA	3.4	1.5	0.7	1.8	4.4	0.8	1.1	1.0	1.7
SA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EC	1.3	1.9	0.4	0.7	3.1	0.9	0.8	0.2	0.8
PCA	1.4	0.5	0.4	1.1	1.4	0.2	1.2	0.2	0.6
Mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FA	0.5	0.5	0.5	0.6	0.3	0.4	0.6	0.2	0.2
TPd	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.2
Qg	0.4	0.0	0.0	0.0	2.3	0.0	0.0	1.5	0.0
IrKg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CPd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
My	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Re	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.2
Q	0.0	0.0	0.2	0.3	0.7	0.0	0.0	0.0	0.0
CR	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ka	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ir	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUGARS									
F	834	769	231	884	7395	316	577	391	607
G	404	455	340	313	632	416	1013	399	466
pH	3.31	3.55	3.49	3.56	3.19	3.29	3.44	3.17	3.39

Table B2(continued): Quantitative results obtained for the white wines.

Compound	CC Chard 99	CC Chard 00	CC Chard 01
ACIDS			
TA	1419	1144	882
MA	584	936	1661
CAc	0	75	0
SAc	424	445	439
AA	608	564	380
LA	2365	1803	1828
PHENOLS			
GA	3.7	4.1	3.6
PrCA	2.7	2.5	1.8
CTA	6.1	12.5	6.3
C	3.6	6.8	7.2
CMA	2.3	2.7	1.3
VA	0.0	0.0	0.0
CA	2.6	3.7	8.0
SA	0.0	0.0	0.0
EC	2.0	6.6	3.2
PCA	1.5	2.4	3.3
Mg	0.0	0.0	0.0
FA	0.3	0.3	0.7
TPd	0.0	0.0	0.0
Qg	0.0	0.8	1.0
IrKg	0.0	0.0	0.0
CPd	0.0	0.0	0.0
My	0.0	0.0	0.0
Re	0.0	0.1	0.1
Q	0.0	0.0	0.2
CR	0.0	0.0	0.0
Ka	0.0	0.0	0.0
Ir	0.0	0.0	0.0
SUGARS			
F	980	1625	1248
G	933	975	847
pH	3.43	3.44	3.44

Table B2(continued): Quantitative results obtained for the white wines.